Role of Proline in Tobacco Cultured Cells Under Arsenate Stress

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Mst. Nur-E-Nazmun Nahar

Graduate School of

Environmental and Life Science

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OKAYAMA UNIVERSITY, JAPAN

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Mst. Nur-E-Nazmun Nahar

Department of Biofunctional Chemistry

Graduate School of Environmental and Life Science

Okayama University, Japan

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ABBREVIATIONS USED

LS, Linsmaier and Skoog

ROS, reactive oxygen species

GSH, reduced glutathione

GSSG, oxidized glutathione

AR, arsenate reductase

SOD, superoxide dismutase

P5C, pyrroline-5-carboxylate

GSA, glutamate-5-semialdehyde

P5CDH, pyrroline-5-carboxylate dehydrogenase

PCs, phytochelatins

ATP, Adenosine tri-phosphate

O_2^-, superoxide anion

FW, Fresh weight

DW, Dry weight

CHAPTER 1

General Introduction

1.1 Toxicity of heavy metals

Heavy metals are metallic elements which are toxic and have a high density (< 5.0 g/cm^3), specific gravity or atomic weight. Arsenic, chromium, cadmium, mercury, and lead have the greatest potential to cause harm because of their extensive use, and widespread distribution in the environment (Baird and Cann, 2012). The toxicity of heavy metals observed in plants when they are present in excessive amounts due to a range of interactions at the cellular level. Enzymes are one of the main targets of heavy metal in plants, and prolonged exposure to heavy metals results in remarkable decreases in enzyme activity (Tyler et al., 1989). Heavy metals can pollute air, water, and soil quality, and cause toxicity to plants and animals (Stankovic and Stankocic, 2013). To maintain the level of essential metals within physiological ranges, plants have evolved a variety of defense mechanisms that control the uptake, accumulation and detoxification of metals.

1.2 Sources of arsenic

The natural source of arsenic is the earth crust. Arsenic can be introduced into the environment from various sources. The sources of arsenic include both natural and anthropogenic (e.g. use of insecticides, herbicides, and phosphate fertilizers) (Cozzolino et al., 2010; Verbruggen et al., 2009) processes. The involvement of arsenic with agricultural practices began after the green revolution when the use of pesticides and fertilizers increased rapidly. Arsenic also finds its way into the food chain (Meharg, 2004). One of the most widespread problems of naturally occurring arsenic is due to leaching into drinking water aquifers, which have been reported in many countries including India and Bangladesh

(Kim et al., 2009). In Bangladesh, arseno pyrite has been identified as the prime source of arsenic pollution (Fazal et al., 2001b).

1.3 Arsenic Transport and Hyperaccumulation in Plants

The processes of heavy metal transport have been recognized as a central mechanism of metal detoxification and tolerance. Transporters play a critical role in arsenic metabolism in plants. Arsenate is the dominant species in aerobic soils and readily enters plant roots via phosphate transporters. A number of the aquaporin nodulin26-like intrinsic proteins are able to transport arsenite, the predominant form of arsenic in reducing environments. Arsenic is primarily taken up by plants via root and leaves. In most plants, arsenic is accumulated through roots because its low mobility restricts its root-to-root translocation, except in arsenic hyperaccumulators (Raab et al., 2005). A “hyperaccumulators” plant is defining as the plant that could tolerate and accumulate >1 mg metal g^{-1} dry mass (Brooks et al., 1977). Three main forms of arsenic in soil are available to plants, namely arsenate (AsO_4^-), arsenite (AsO_3^-) and methylated arsenic. To understand how plant take up and metabolize arsenic is important for the mitigation. Plant roots selectively take up arsenic species via distinct pathways and transporters. Hyperaccumulators such as *Pteris vittata* L, *Agrostis tenerrima Trin* have an extraordinary ability to absorb heavy metals from the soil under varying concentration of heavy metals (Ma et al., 2001).

1.4 Arsenic toxicity

Arsenic is a highly toxic metalloid. It is naturally taken up by plants from the water and soil. Arsenic toxicity to living cells causes at very low concentration and its toxicity symptoms in plants range from growth inhibition to ultimate cell death (Barrachina et al., 1995). Indeed, arsenic toxicity in humans has recently become evident on a very large scale in Bangladesh (Dhar et al., 1997). The acceptable level of arsenic safe drinking water is 0.01 mg/L (WHO,

2003). Plants can respond to arsenic toxicity by a variety of mechanisms such as hyperaccumulation, antioxidant defense system, and phytochelation (Garg and Singla, 2011). Arsenic interacts with sulfhydryl (-SH) groups of enzymes and proteins, leading to inhibition of several important cellular functions (Meharg and Hartley Whitaker, 2002). Plant growth can severely have inhibited through exposure of arsenic by slowing or arresting expansion and biomass accumulation. The toxicity of arsenic cause degradation of plant reproductive capacity through losses in fertility, yield, and fruit production. Arsenic interferes with the critical metabolic processes of plants that can lead to the death of plant (Garg and Singla, 2011).

1.5 Cell death

Cell death is essential for growth and development of eukaryotes, by maintaining tissue and organ homeostasis in concert with proliferation, growth, and differentiation (Breusegem and Dat, 2006). Treatment of tobacco cells in suspension cultures with arsenic caused cell death. There are two main categories of cell death such as apoptosis and necrosis. The difference between these two forms was based on the presence or absence of specific biochemical and molecular hallmarks, such as DNA laddering, cytochrome c, caspase involvement, ATP depletion, cytoplasmic swelling, and loss of membrane integrity (Pennell and Lamb, 1997).

1.6 DNA fragmentation

DNA fragmentation is an important hallmark of cell death. Arsenic caused DNA damage by attacking on its bases and sugar moieties, consequently DNA fragmentation. It has already been documented that arsenic can replace phosphorous from phosphate group of DNA and inhibits DNA repair system which could lead to genomic template instability (Erturk et al., 2015). NaCl-induced apoptosis-like cell death along with genomic DNA degradation was also observed in tobacco protoplasts (Lin et al., 2006).

1.7 Reactive oxygen species

Reactive oxygen species (ROS) are produced as a normal product of plant cellular metabolism. Despite their destructive activity, ROS are well-described second messengers in a variety of cellular processes including tolerance to environmental stresses (Desikan et al., 2001; Yan et al., 2007). The most common ROS include singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH). ROS are always formed by the inevitable leakage of electrons onto O_2 from the electron transport activities of chloroplasts, mitochondria, and plasma membranes or as a by-product of various metabolic pathways localized in different cellular compartments. Production and removal of ROS must be strictly controlled in order to avoid oxidative stress. When the level of ROS exceeds the defense mechanisms, a cell is said to be in a state of “oxidative stress ”. However, the equilibrium between production and scavenging of ROS is perturbed under a number of stressful conditions such as salinity, drought, toxicity due to metals, pathogens, and so forth. Enhanced level of ROS can cause damage to biomolecules such as lipids, proteins and DNA.

1.8 Oxidative damage and antioxidant defense mechanism

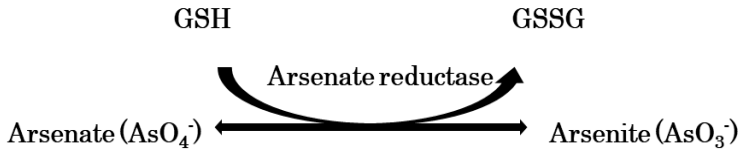
In the cell, arsenate can be readily converted to arsenite that is more toxic. Arsenate and arsenite disrupt plant metabolism through distinct mechanisms. Arsenite is a dithiol reactive compound that binds to and potentially inactivates enzymes containing closely spaced cysteine residues or dithiol cofactors. Arsenic exposure generally induces the production of ROS that can lead to the production of antioxidant metabolites and numerous enzymes involved in antioxidant defense mechanisms. Oxidative carbon metabolism, amino acid and protein relationships, and nitrogen and sulfur assimilation pathways are also impacted by arsenic exposure (Finnegan and Chen, 2012). Metal toxicity can cause a redox imbalance and induce the increase of ROS concentration, activating the antioxidant defense mechanisms of plants (Sharma and Dietz, 2009).

1.9 Glutathione

Glutathione (γ-glutamyl-cysteinyl-glycine) represents the major pool of reduced sulfur (Kurnert & Foyer 1993). Under normal conditions, glutathione is predominantly present in its reduced form (GSH), with only a small proportion present in its fully oxidized state (GSSG). Glutathione play roles in biosynthetic pathways, detoxification, antioxidant biochemistry and redox homeostasis. Glutathione is the precursor of phytochelatins (PCs) compounds that are synthesized in response to heavy metals. Arsenate and arsenite are also known to have a high affinity for thiols such as glutathione (Jocelyn 1972). Glutathione is a key ROS scavenger and major cellular redox buffer; it is a crucial part of stress signaling pathways and has important roles in the regulation of the cell cycle. Glutathione is the main non-protein thiols of the cell and is a non-enzymatic antioxidant that participates of free radical scavenging and modulation of the cellular redox status and thiol-disulfide status of proteins (Cnubben et al., 2001; Foyer and Noctor, 2011).

1.10 Arsenate reductase

Arsenate reductase (glutaredoxin) (EC 1.20.4.1) catalyzes the following chemical reaction-



Arsenate reductase (AR) belongs to the family of oxidoreductases. This group of arsenate reductases belongs to the low-molecular weight protein-tyrosine phosphatase family, as does a group of glutathione/glutaredoxin type arsenate reductases. Arsenate can be reduced to arsenite nonenzymatically, but the process is too slow to be physiologically significant (Bhattacharjee et al., 1999). The thiol-linked reductases are required to confer resistance to arsenate in both prokaryotes (Ji et al., 1994) and eukaryotes (Bobrowicz et al.,

1997). The reduction of arsenate to arsenite is catalyzed by AR which is also considered as a mechanism involved in detoxification because arsenite can bind with PCs. Arsenate reduction caused by AR and GSH serving as the electron donor (Ellis et al., 2006).

1.11 Superoxide dismutase (SOD)

It is a group of metalloisozymes that neutralized the highly reactive superoxide radical into O_2 and H_2O_2 so it can play a very important role in the protection of cells upon stress (Fridovich 1995).

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \Longrightarrow H_2O_2 + O_2$$

Superoxide dismutase catalyzes the dismutation of the O_2^- into O_2 or H_2O_2. Superoxide dismutase constitutes the first line of defense against ROS within a cell (Alscher, 2002). Superoxide dismutase is an antioxidant enzyme associated with metal cofactors. Based on the metal co-factor used by the enzyme, SODs are classified into three groups: iron SOD (Fe SOD), manganese SOD (Mn SOD), and copper-zinc SOD (Cu-Zn SOD). During arsenic stress, the up regulation of Cu/Zn SOD has been reported in rice seedlings (Shri et al. 2009). The proteomic analysis of maize root reveals that Cu/Zn SOD is one of the highly responsive enzymes to arsenic which involved in cellular homeostasis during redox disturbance (Requejo and Tena, 2005). Mylona et al. (1998) demonstrated that SOD activity increased in response to low arsenic concentration but a high concentration of arsenic inhibits the accumulation of SOD mRNA and leads to decline its activity.

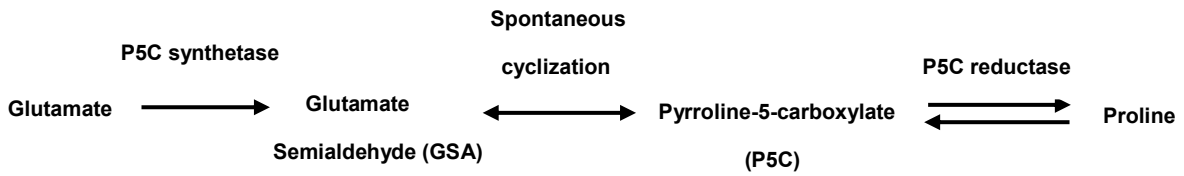
1.12 Compatible solutes

Compatible solutes or osmoprotectants are small molecules that act as an osmolyte and help organisms to survive at extreme osmotic stress. Compatible solutes are low molecular weights, highly soluble organic compounds that are usually non-toxic at high cellular

concentrations. These solutes provide protection to plants from stress by contributing to cellular osmotic adjustment, ROS detoxification, protection of membrane integrity and enzymes/protein stabilization (Yancey,1994; Ashraf and Foolad, 2007). In plants, the accumulation of compatible solute is increased. Examples of compatible solutes include amino acids, glycine betaine etc. These molecules accumulate in cells and balance the osmotic difference between the cells surroundings and the cytosol. Compatible solutes have also been shown to play a protective role by maintaining enzyme activity.

1.12.1 Proline

Proline is one of the most accumulated compatible solutes found in plants as well as in other organisms. Most plant species can accumulate proline in response to environmental stresses including heavy metal stress. Proline is predominantly synthesized from glutamate. Based on its known properties proline may be involved in plant heavy metal stress by different mechanisms, *i.e.* osmo- and redox-regulation, metal chelation, and scavenging of free radicals. However, in some cases, exogenous proline showed its toxicity to plants and caused programmed cell death (Hellmann et al., 2000). Osmoregulation appears to be a common element of plant reactions to various abiotic stress. Stress conditions up-regulate the key enzyme ∆1-pyrroline-5-carboxylate synthetase (P5CS), for proline biosynthesis (Hare et al., 1999) that catalyzes the first two steps of proline biosynthesis from glutamate in plants (Delauney and Verma, 1993). Proline plays multiple roles in plant stress tolerance.



Free proline accumulates in plants exposed to Cd (Sharma and Dietz, 2006). Exogenous

application of proline mitigates salt stress (Okuma et al., 2000, 2004; Hoque et al., 2007) and cadmium stress (Islam et al., 2009) in BY-2 cells.

1.12.2 Arginine

L-arginine is an important and unique amino acid in plants. It is one of the most functionally diverse amino acids in living cells and also serves as a precursor for the synthesis of protein, nitric oxide, creatine, polyamines, agmatine, and urea (Sidney and Morris, 2007). The arginine metabolism plays important role in perception and adaptation of plant to environmental disturbances.

1.12.3 Alanine

The application of amino acids can result in a better plant development since these molecules can act as signals of several beneficial physiological processes of plants. Alanine, unlike proline, follows the separate catabolic pathway. Alanine inhibits arginine degradation process and formation of urea from arginine.

1.12.4 Betaine

Glycine betaine (Betaine), a compatible solute, is a small organic metabolite that are very soluble in water and non-toxic at high concentrations. Betaine is involved in the protection of macro components of plant cells, such as protein complexes and membranes, under stress conditions. Among the compatible solutes, betaine is particularly effective protectant against abiotic stress (Sakamoto and Murata, 2000;2001). Exogenous application of betaine can improve the tolerance of numerous plant species to various types of abiotic stresses. In addition to its functions as an osmoprotectant, betaine contributes to stabilization and protection of membranes, proteins and enzymes against stresses (Papageorgiou and Murata, 1995; Ashraf and Foolad, 2007).

1.13 Lipid peroxidation

Lipid peroxidation is one of the first consequences of oxidative damage. Malondialdehyde (MDA), a lipid peroxidation product, has widely been used to assess the levels of free radicals in living cells. The concentration of MDA was used as an indicator of lipid peroxidation. The restriction on nutrient uptake can be associated to increased lipid peroxidation, which reduces its functionality.

1.14 Arsenic tolerance mechanisms

Though arsenic is not a redox metal, there is significant evidence that exposure of plants to inorganic arsenic results in the generation of ROS. Plants have evolved mechanisms to protect cells from the effects of ROS by using enzymatic antioxidants such as SOD, catalase (CAT), and ascorbate peroxidase and non-enzymatic antioxidants such as ascorbate, glutathione, and α-tocopherol (Sairam et al., 2005; Gunes et al., 2009). Glutathione has a dual role in response to metal stress, and it has been suggested that PCs production, resulting in oxidized glutathione depletion, could itself cause oxidative stress (Hartley-Whitaker et al., 2001; Verbruggen et al., 2009). One of the major types of arsenic tolerance/resistance mechanisms that have been demonstrated in plants is complexation of metals by PCs. Arsenate can be readily reduced to arsenite via arsenate reductase in a glutathione-dependent reaction (Mukhopadhyay et al., 2000) and arsenite can subsequently complex with thiols, particularly PCs. One potential strategy for plants to protect from the toxicity of arsenic to methylate inorganic arsenic to organic arsenic species (Lomax et al., 2012; Zhao et al., 2013).

1.15 Purpose of the study

To investigate the role of exogenous proline in plants, the present research was conducted with the following objectives:

(i) To investigate the effects of proline on arsenic-induced cell growth inhibition in tobacco BY-2 cultured cells.

(ii) To clarify the protective mechanisms of proline in the components of the antioxidant defense systems against arsenic-induced oxidative damage.

(iii) To compare the role of proline with other osmolytes such as arginine, alanine, glutamate, and betaine on BY-2 cell growth under arsenate stress.

CHAPTER 2

Regulation of arsenate-induced changes by exogenous proline in tobacco BY-2 cells

2.1 ABSTRACT

Arsenic, a non-essential toxic element, causes toxicity to plants. Plants take up arsenic mainly as arsenate. Proline is accumulated as a compatible solute in plants under various stress conditions. Exogenous proline scavenges free radicles, improves plant metabolism and stimulates plant growth under stress conditions. However, in some cases, exogenous proline showed its toxicity to plants and caused programmed cell death. It is reported that proline ameliorates heavy-metal toxicity in plants. However, the role of proline in arsenate-stressed BY-2 cells remains unclear. In this study, I investigated the effects of exogenous proline on tobacco BY-2 cells cultured under AsO_4^- stress and found that proline depending on its concentrations plays dynamic roles in BY-2 cells such as mitigation of arsenate stress in response to lower concentrations of proline (e.g., 0.05 mM), whereas sensitization of BY-2 cells to arsenate in response to higher proline treatment (e.g., 10 mM). In this study, the effects of exogenous proline, exogenous arsenate, and the co-treatment of arsenate and proline on BY-2 cells growth, cell death, and cell number were presented. Here, AsO_4^- significantly inhibited the growth of BY-2 cells at 60 μM but not at either 40 μM or 50 μM. Proline at 0.05 mM to 10 mM did not affect the cell growth but delayed it at 20 mM. Therefore, for mitigating the arsenic stress, I examined the effects of a wide range (0.05, 0.1, 0.5, 1, and 10 mM) of exogenous proline on the inhibition of cell growth by 60 μM arsenate and found that proline at 0.05 mM and 0.1 mM alleviated the arsenate-induced cell growth inhibition, but surprisingly accelerated the growth inhibition at 1 mM and 10 mM. Proline at 0.05 mM and 0.1 mM significantly decreased the number of Evans Blue stained cells but 10

mM proline boosted the number of stained cells. Proline at 0.05 mM and 0.1 mM increased the total number of cells, whereas 10 mM proline decreased the total number of cells. These results indicate that the effects of 0.05 mM and 0.1 mM proline on arsenate-stressed BY-2 cells reversed with the increase of proline concentration to 10 mM, and also suggests that the lower concentration of proline mitigates arsenate stress, whereas the higher concentration of proline sensitizes BY-2 cells to arsenate. To insight into the issue that 10-mM proline enhances the sensitivity of BY-2 cells to arsenate, I further investigated the effects of 10 mM proline on BY-2 cells treated with 40 μM arsenate and found that 40 μM arsenate did not inhibit cell growth in the absence of proline but inhibits it in the presence of proline, suggesting that application of 10 mM proline enhances the adverse effects of arsenate. Together, these results suggest that proline plays two distinct roles in BY-2 cells in the presence of arsenate.

2.2 INTRODUCTION

Arsenic, a toxic metalloid, is widely distributed in the environment and causes physiological and structural disorders in plants (Sharma, 2012). Arsenic accelerates cell death and inhibits plant growth (Stoeva and Bineva, 2003; Stoeva et al., 2005). Now a day, the reduction of crop yield by arsenic stress has been recognized as a threat to the sustainable food production (Brammer and Ravenscroft, 2009; Panaullah et al., 2009). Arsenic occurs predominantly as inorganic forms such as arsenate (AsO_4^-) and arsenite (AsO_3^-). Plants take up arsenic mainly as arsenate (Tripathi et al., 2007).

The organic compatible solute, proline, has been reported to accumulate in plants subjected to various abiotic stresses such as salt stress (Sakamoto and Murata, 2000). Exogenous proline functions as a free radical scavenger and an enzyme protectant (Tsugane et al., 1999; Hong et al., 2000). Okuma et al. (2004) reported that proline exhibits an antioxidant activity which was proved by the 1,1-diphenyl-2-picrylhydrazyl assay. Proline

improves plant metabolism and stimulates plant growth under stress conditions (Alia et al., 1991; Fedina et al., 1993). Earlier studies suggest that exogenous application of proline confers protection against metals (Islam et al., 2009) and that proline play diverse roles in plants and confer protection against a variety of abiotic stresses (Hare et al., 1998). However, in some cases, exogenous proline showed its toxicity to plants (Hellmann et al., 2000) and caused programmed cell death by producing ∆1-pyrroline-5-carboxylate (P5C), an intermediate in biosynthesis and degradation of proline, is assumed to play a role in cell death in plants (Deuschle et al., 2001).

Proline has been known to accumulate under metal stress in plants (Sharma and Dietz, 2006; Xu et al., 2009). In this study, I investigated the effects of exogenous proline on tobacco BY-2 cultured cells under arsenate-stress conditions. I found that arsenate inhibited the BY-2 cell growth. Exogenous proline at lower concentration mitigates arsenic induced growth inhibition by decreasing Evans Blue stained cells and increasing the total number of cells, whereas, that arsenate accelerated the inhibition of cell growth in the presence of higher concentration of proline. I also found that arsenate boosted the number of dead cells and decreased the total number of cells in the presence of higher proline. These results indicated that exogenous proline at higher concentration did not mitigate arsenate stress in BY-2 cells but that proline enhances the sensitivity of BY-2 cells to arsenate.

2.3 MATERIALS AND METHODS

2.3.1 Culture of tobacco BY-2 cells

Suspension-cultured cells of tobacco (*Nicotiana tabacum* L., cv. BY-2) were used for the arsenic-unadapted cell lines (Murata et al., 1994a, b). The modified LS medium (Linsmaier and Skoog, 1965) was used as a standard medium in which KH_2PO_4 and thiamine-HCl were increased to 370 and 1 mgL^{-1}, respectively, supplemented with 3% sucrose and 1 μM 2,4-

dichlorophenoxyacetic acid (Nagata et al., 1981). The LS medium supplemented with 40 μM, 50 μM, and 60 μM AsO_4^- were regarded as the standard arsenic medium of 40 μM, 50 μM, and 60 μM AsO_4^-, respectively. The 0.05 mM, 0.1 mM, 0.5 mM, 1 mM and 10 mM proline media were the 60 μM AsO_4^- media containing 0.05 mM, 0.1 mM, 0.5 mM, 1 mM and 10 mM proline. The 0.5 mM, 1 mM and 10 mM proline media were the 40 μM AsO_4^- media containing 0.5 mM, 1 mM and 10 mM proline. The BY-2 cells were cultured and maintained as described previously (Murata et al., 1994a, b). The cells were sub-cultured weekly and were incubated on a rotary shaker at 100 rpm at 25°C in the dark.

2.3.2 Measurement of BY-2 cell growth

The growth of BY-2 cells was measured as described previously (Murata et al., 1994a, b). To measure the BY-2 cell growth, the cells were incubated in the culture media for different days such as 2, 4, 6, 8, and 10 days. After incubation, the cells were collected by removing the aqueous solution in a vacuum using a nylon sieve (pore size 45 μm), and the fresh weight (FW) of the cells was taken. Then the cells were dried in an oven at 70°C and the dry weight (DW) was measured. Both the FW and DW of the cells were taken at different days after inoculation (DAI).

2.3.3 Estimation of cell death of BY-2 cells

Dead cells were quantified by the method described previously (Yano et al., 1998; Takatsuka et al., 2004). Cells were stained with Evans Blue solution (0.05%) for 20 min and subsequently washed with distilled water to remove the excess dye. Dye that had bound to dead cells was solubilized in 1 mL of 50% methanol that contained 1% sodium dodecyl sulfate followed by the incubation for 50 min at 50°C. Then the absorbance was measured at 600 nm by a spectrophotometer. For calculation, the cells prepared in the same way were subjected to two cycles of freezing at -20°C and thawing at room temperature. The cells

killed in such a way were used to define 100% cell death. The value obtained from 4-day-old cultured cells was defined as 0% cell death. The death cells were calculated by comparing the absorbance of the samples with the absorbance of 100% cell death.

2.3.4 Counting of BY-2 total cell number

The number of total cells was counted using Haemacytometer (Burker-Turk, 0.0025 mm^2, 0.004 mm^2) under the microscope.

2.3.5 Statistical analysis

Unless stated otherwise, the significance of differences between the mean values of all parameters was assessed by Tukey's test. Differences at the level of $p \leq 0.05$ were considered as significant.

2.4 RESULTS

2.4.1 Effects of exogenous proline on BY-2 cell growth in the absence of arsenate

To investigate the roles of exogenous proline for the mitigation of arsenate stress in BY-2 cells, we examined whether exogenous proline shows any effects on BY-2 cells. We measured the FW and DW of cells at 0, 2, 4, 6, 8, and 10 DAI in response to exogenous proline in the absence of arsenate. We found that 0.05 mM, 0.1 mM, 0.5 mM, 1 mM and 10 mM proline did not change BY-2 cell growth compared with control but delayed it at 20 mM as well as the cell growth curve was dramatically increased at 4 to 6 DAI and then steadily increased up to 10 DAI (Fig.2.1A and B).

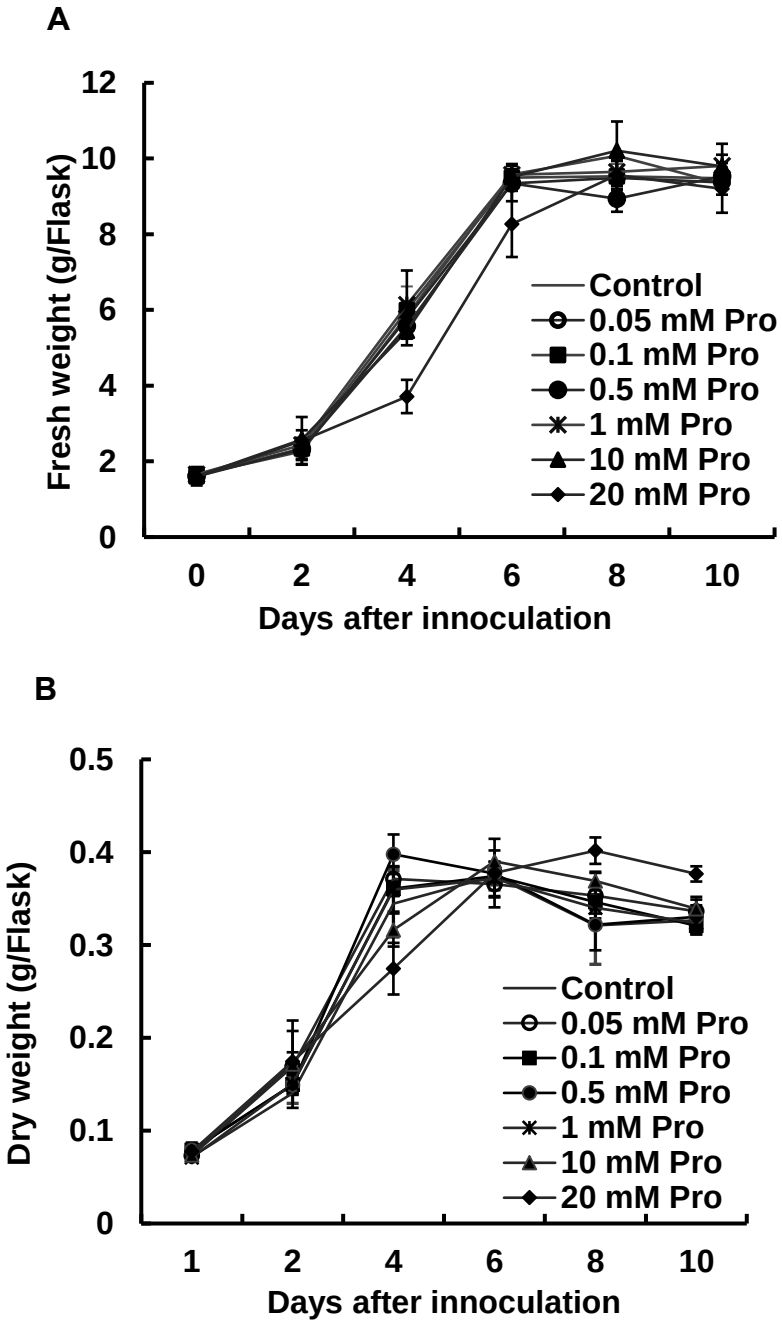


Figure 2.1 Effects of exogenous proline (Pro) on BY-2 cell growth. A, Shows the cell growth based on fresh weight and B, shows the cell growth based on dry weight in response to 0.05 mM, 0.1 mM, 0.5 mM, 1 mM, 10 mM and 20 mM Pro at 0, 2, 4, 6, 8, and 10 DAI. Averages of cell growth from three independent experiments (n = 3) are shown. Error bars represent SE. There were no significant differences ($p<0.05$) between control (untreated) cells and treated cells in fresh weight or dry weight at each time point after inoculation.

2.4.2 Effects of exogenous arsenate on BY-2 cell growth

We tested the effects of arsenate on BY-2 cell growth. We measured the FW and DW of BY-2 cells at 0, 4, 6, and 8 DAI in response to 40 µM, 50 µM, and 60 µM AsO_4^-. Compared with control, arsenate did not affect the growth of BY-2 cells at 40 µM and 50 µM but significantly inhibited it at 60 µM at all DAI (Fig.2.2A and B).

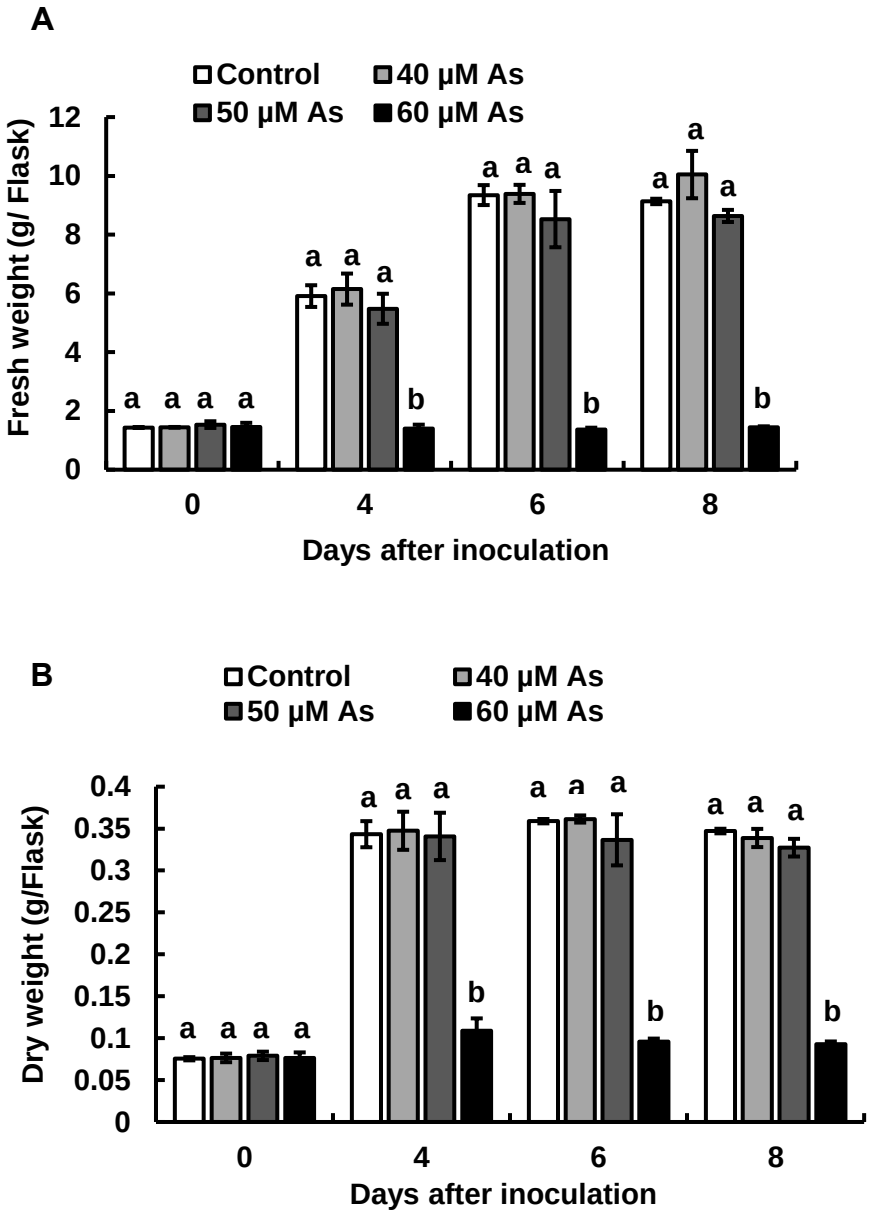
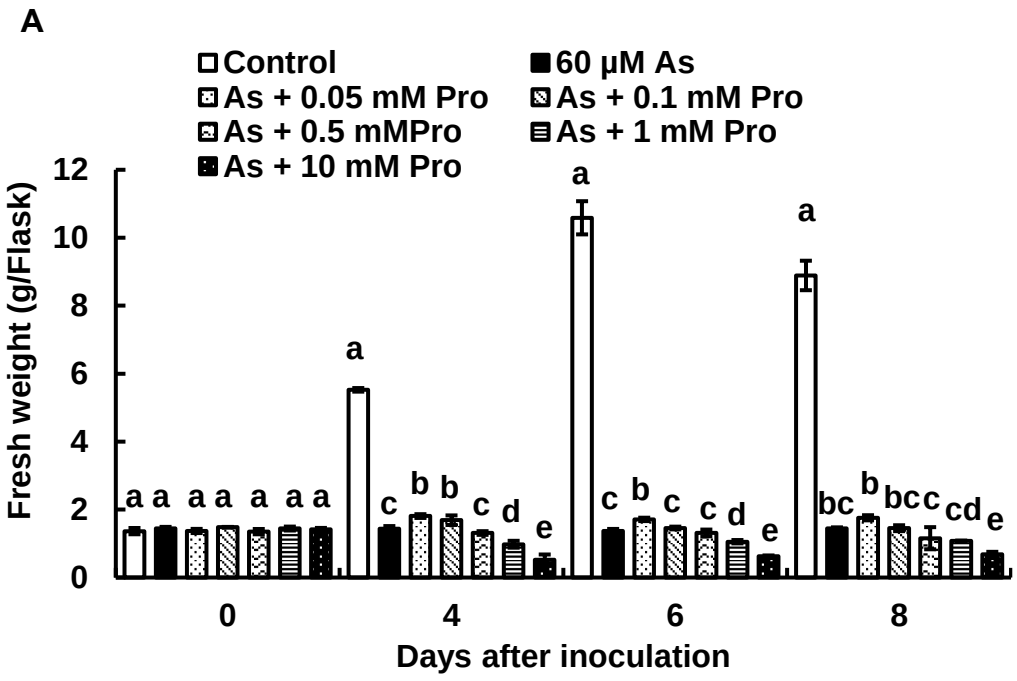


Figure 2.2 Effects of exogenous arsenate (As) on BY-2 cell growth. A, Shows the cell growth based on fresh weight and B, shows the cell growth based on dry weight in response to 40 µM, 50 µM and 60 µM arsenate at 0, 4, 6 and 8 days after inoculation. Averages of cell

growth from three independent experiments (n = 3) are shown.

2.4.3 Effects of exogenous proline on the inhibition of BY-2 cell growth by arsenate

To investigate whether exogenous proline recovered the inhibition of cell growth by arsenate, we examined the effects of exogenous proline on the growth of BY-2 cells cultured at 40 μM AsO_4^- and 60 μM AsO_4^-. The FW and DW of cells at 0, 4, 6, and 8 DAI were measured. At 60 μM AsO_4^- stress condition, 0.05 mM proline significantly recovered the AsO_4^- induced inhibition of BY-2 cell growth, whereas 0.1 mM and 0.5 mM proline did not affect the cell growth but 1 mM and 10 mM proline significantly inhibited it (Fig. 2.3A and B). Moreover, AsO_4^- at 60 μM induced more inhibition of cell growth in the presence of 1 mM and 10 mM exogenous proline than in the absence of exogenous proline. In the presence of 40 μM AsO_4^-, neither 0.5 mM proline nor 1 mM proline affected the cell growth but 10 mM proline inhibited it (Fig. 2.4A and B). These results suggest that exogenous proline at lower concentration mitigate the arsenate-induced growth inhibition of BY-2 cells but did not at 1 mM and 10 mM and further enhances the sensitivity of BY-2 cells to arsenate.



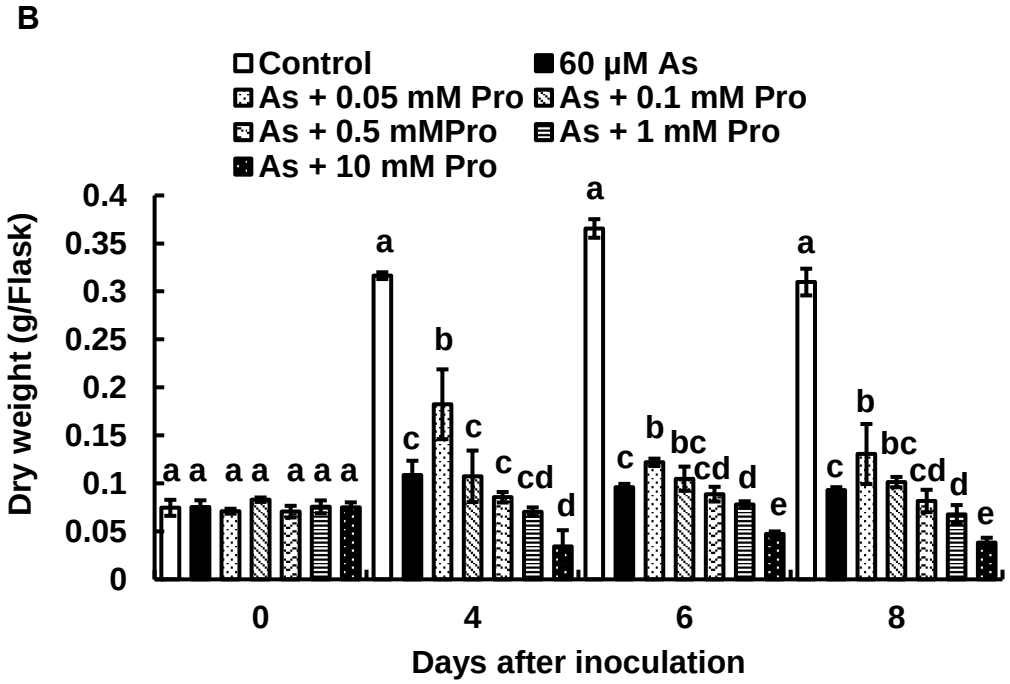


Figure 2.3 Effects of exogenous pro (Pro) on 60 μM arsenate (As)-induced growth inhibition of BY-2 cells. Increment of cell growth in the presence of 0.05 mM proline and enhancement of arsenate-induced cell growth reduction (Fresh weight basis, A; dry weight basis, B) in the presence of both the 1 mM and 10 mM Pro but not in the presence of 0.1 mM and 0.5 mM Pro at 4, 6 and 8 days after inoculation. Averages of cell growth from three independent experiments (n = 3) are shown. The error bars represent SE. For the same inoculation day, values indicated by the same letter do not differ significantly at 5% level of significance as determined by Tukey's test.

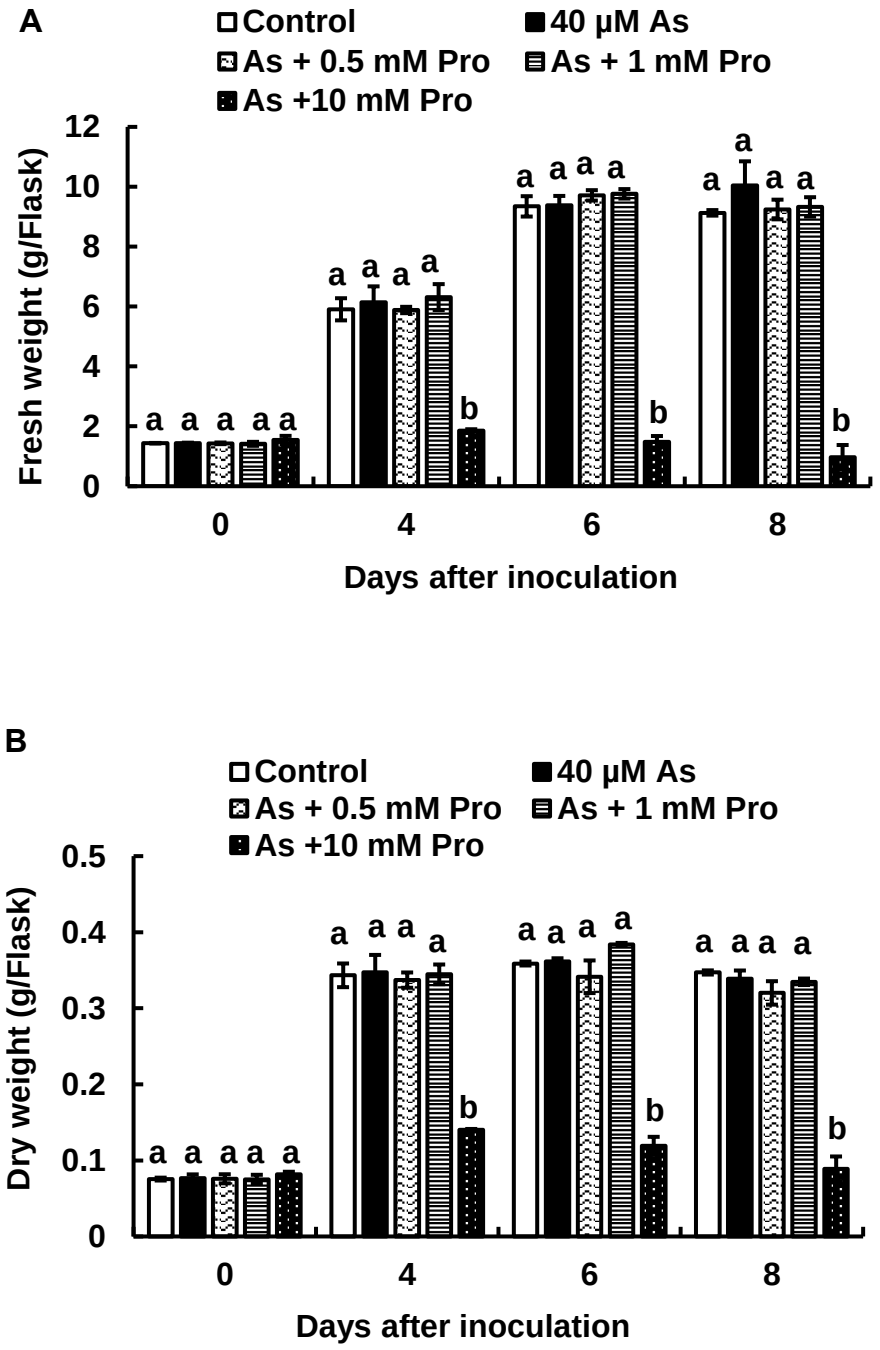
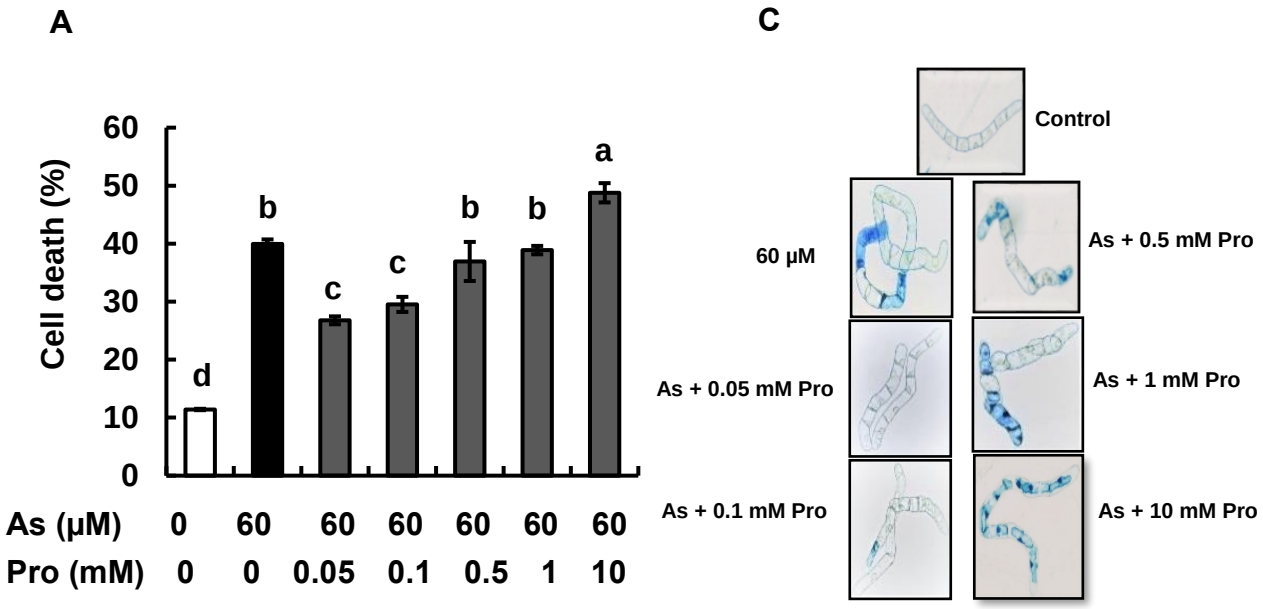


Fig. 2.4 Effects of exogenous proline (Pro) on 40 μM arsenate-stressed BY-2 cells. Reduction of BY-2 cell growth by arsenate (FW basis, A; DW basis, B) in the presence of 10 mM Pro but not in presence of 0.5 mM or 1 mM Pro at 4, 6 and 8 DAI. Averages of cell growth from three independent experiments are shown. Error bars represent SE. For the same inoculation day, values indicated by the same letter do not differ significantly at 5% level of significance as determined by Tukey's test.

2.4.4 Effects of exogenous proline on arsenate-induced cell death

In this study, we examined the cell death of BY-2 by arsenate in the presence and absence of proline. Arsenate at 60 μM increased the number of Evans blue stained cells by 25% compared with control. In the presence of AsO_4^-, 0.05 mM and 0.1 mM exogenous proline significantly recovered the AsO_4^- induced cell death. On the other hand, arsenate boosted the number of stained cells by 45% in the presence of 10 mM proline but not in the presence of either 0.5 mM or 1 mM proline (Fig. 2.5A). Arsenate at 40 μM did not show any effect on the cell death whereas that arsenate in the presence of 10 mM proline significantly increased the Evans blue positive cells but not in the presence of 0.5 mM and 1 mM proline (Fig. 2.5B). These results indicate that application of proline recovered the arsenate-induced cell death at lower concentration but increase the number of dead cell by arsenate at higher concentration.



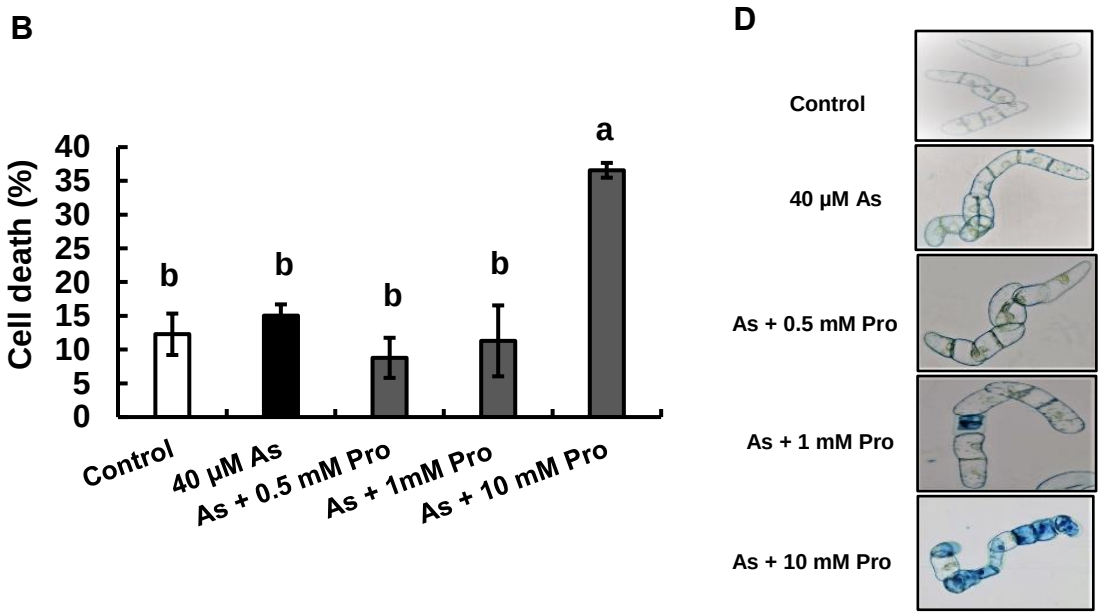
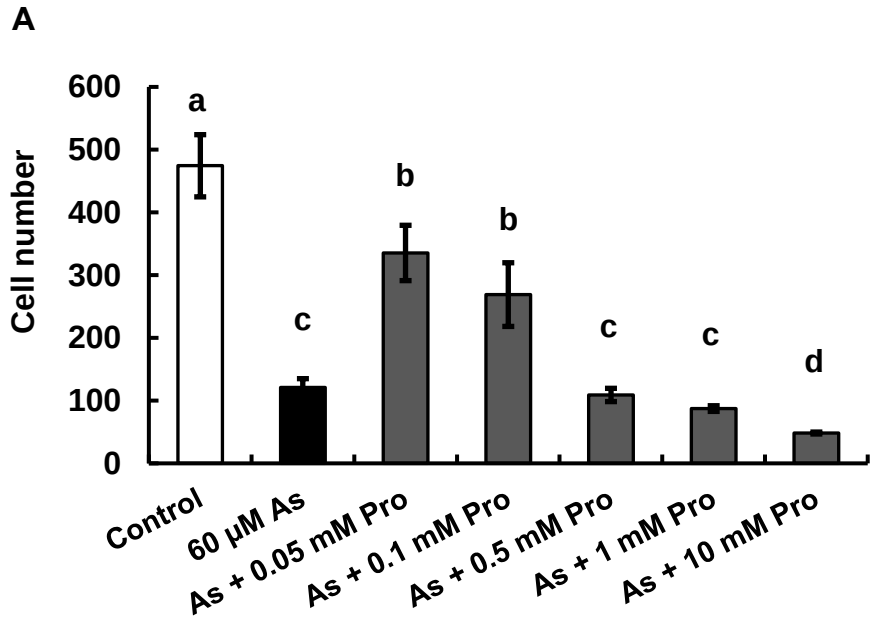


Figure 2.5 Effects of exogenous proline (Pro) on arsenate (As)-induced BY-2 cell death. A, Induction of cell death by the co-treatment of 60 μM arsenate. Arsenate at 60 μM induced significant cell death which was recovered by 0.05 mM and 0.1 mM Pro, and enhanced in the presence of 10 mM Pro. B, Induction of cell death by the co-treatment of 40 μM arsenate and 10 mM Pro but not by the individual treatment of 40 μM arsenate. Averages of cell death from three independent experiments (n = 3) are shown. The error bars represent SE. Values indicated by the same letter do not differ significantly at 5% level of significance as determined by Tukey's test. C and D, Evans Blue staining of arsenate-treated BY-2 cells in the presence or absence of Pro. At 60 μM arsenate-stress condition, BY-2 cells showed more Evans Blue positive cells compared with control. Pro at 0.05 mM and 0.1 mM decreased the Evans Blue stained cells at AsO_4^- stress but the stained cell was enhanced in the presence of 10 mM Pro (Fig. 2.5C). At 40 μM arsenate-stress condition, BY-2 cells showed Evans Blue-stained cells in the presence of Pro but not in the absence (Fig. 2.5D).

2.4.5 Effects of exogenous proline on the reduction of BY-2 cell number by arsenate

In our study, we monitored the effects of exogenous arsenate at 40 μM and 60 μM on the total number of BY-2 cells with or without the application of proline. We found that arsenate at 60 μM decreased the total number of cells by 3.5-fold compared with control. At 60 μM arsenate, proline at 0.05 mM and 0.1 mM significantly increased the total number of cell and that arsenate in the presence of 1 mM and 10 mM proline decreased the number of cells by 4.5- and 7.5-fold, respectively (Fig. 2. 6A). We also found that arsenate at 40 μM did not show any effect on the cell number compared with control whereas that arsenate in the presence of 10 mM proline significantly decreased the cell number but not in the presence of 0.5 mM and 1 mM proline (Fig. 2.6B). These results indicate that application of proline at lower concentration recovered the reduction of cell number by arsenate but higher proline level enhances the arsenate-induced decrease of cells number.



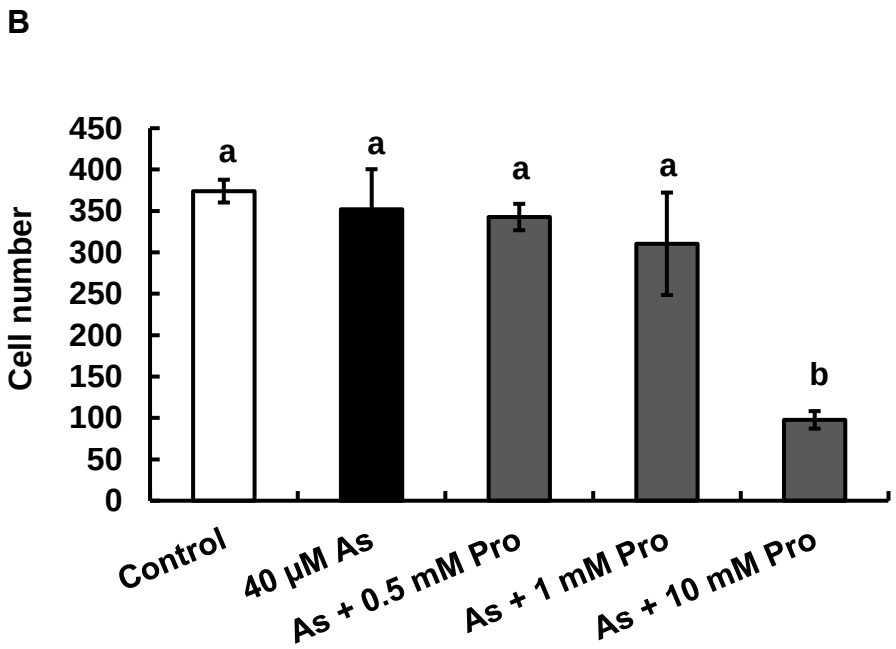


Figure 2.6 Reduction of BY-2 cell number by arsenate (As) in the presence or absence of proline (Pro). A, the reduction of cell number by the co-treatment of 60 μM arsenate and increment of cell number at 0.05 mM, and the reduction in cell number at 10 mM Pro. B, Decrease of cell number by the co-treatment of 40 μM arsenate and 10 mM Pro but not by the arsenate alone. Averages of cell number from three independent experiments (n = 3) are shown.

2.5 DISCUSSION

Arsenic is one of the most hazardous elements in the environment and becomes a global agricultural problem. Accumulation of arsenic in plants causes destruction of cellular membranes interferes with plant metabolic processes and reduces plant productivity (Sharma, 2012; Singh et al., 2006). It was reported that proline ameliorates heavy-metal toxicity in plants. However, whether proline mitigates AsO_4^- stress in BY-2 cells are to be investigated. In this study, we present the AsO_4^--induced growth inhibition of BY-2 cells. Here, the AsO_4^-- induced growth inhibition was recovered by lower concentration of exogenous

proline and the increasing rate of growth inhibition by that arsenate in the presence of higher proline. We also demonstrate that the AsO_4^--induced increment of cell death and the reduction of total cell number was recovered by lower proline but did not in the presence of higher proline.

It is well-known that arsenate inhibits the growth of plants (Shri et al., 2009; Ali et al., 2014). In this study, we found that AsO_4^- significantly inhibited the growth of BY-2 cells at 60 μM but not at either 40 μM or 50 μM (Fig. 2.2A and B). Therefore, for mitigating the arsenic stress, we examined the effects of exogenous proline on the inhibition of cell growth by 60 μM arsenate. Our results indicate that application of proline at lower concentration (0.05 mM) ameliorated the arsenate-induced growth reduction. On the other hand, proline at higher concentration (10 mM proline) did not recover arsenate-induced growth reduction but surprisingly that arsenate enhances the cell growth reduction in the presence of 1mM and 10 mM proline (Fig. 2.3A and B). To insight into this issue, we further investigated the effects of 0.5 mM to 10 mM proline on BY-2 cells treated with 40 μM arsenate. Arsenate at 40 μM did not inhibit cell growth in the absence of proline but inhibits it in the presence of proline (Fig. 2.4A and B), suggesting that application of 10 mM proline enhances the adverse effects of arsenate. In contrast to our proline sensitivity enhancement results, Singh et al. (2015) reported that exogenous proline application ameliorated toxic effects of arsenate in *Solanum melongena* seedlings. This difference may come from the major difference in endogenous proline contents between BY-2 cells (approximately 3 mM) and eggplant seedlings (around 1 μg/g-FW; almost equivalent to about 10 μM). Choudhury et al. (2010) and Siddiqui et al. (2015) reported that arsenate stress increased the proline contents. However, it is unclear which inhibition of plant growth is due to accumulation of arsenate or proline or due to additive or synergistic effect.

In plants, heavy metals can cause tissue damage, growth inhibition, and even death. In this study, we found that arsenate induced the number of Evans Blue-stained (dead) cells

(Fig. 2.5A) and decreased the total number of BY-2 cells (Fig. 2.6A), which is consistent with the previous results (Xue and Yi, 1014). Moreover, we found that exogenous proline at lower concentration recovered the arsenate-induced cell death and increased the total number of cells at arsenate stressed condition. On the contrary, proline at higher concentration boosted the Evans Blue stained cells as well as the reduction of cell number by arsenate is potentiated in response to exogenous proline. These results suggest that arsenate decreased the number of death cell and increases the total number of cell in the presence of lower proline level but increased the cell death and enhances the reduction of total cell number in the presence of higher proline.

There is no clear consensus about the role of proline in plants as well as the mechanism by which proline mitigates heavy metal stresses in plants. Previous research reported that proline mitigates stresses in plants. For example, application of proline ameliorates salt stress (Hoque et al., 2007) and cadmium stress in BY-2 cells (Islam *et al.,* 2009). However, the negative role of exogenous proline was also reported in some cases. For instances, exogenous proline shows toxicity to plants (Hellmann et al., 2000; Deuschle et al., 2001) and proline at 2 mM inhibits the growth of saltgrass (*Distichlis spicata*) (Rodriguez and Heyser, 1988). It was also reported that the accumulation of proline in plants under stress condition is not associated with the mitigation of stress but it is just a symptom (Liu and Zhu, 1997) and did not show any protective value (Moftah and Michel, 1987). In the present study, our data showed that proline ameliorates arsenic stress at lower concentration and higher proline did not mitigate arsenic stress in BY-2 cells but arsenate induced more stressing effects in the presence of higher proline. The previous reports with our findings suggest that the mitigatory role of proline might depend on some conditions such as type of stress, and plant species.

Together, we conclude that exogenous proline at lower concentration mitigates arsenate stress but higher proline enhances the sensitivity of BY-2 cells to arsenate.

CHAPTER 3

Mitigation of arsenate stress by exogenous proline in cultured tobacco cells under arsenate stress

3.1 ABSTRACT

Arsenic, one of the most toxic heavy metals, causes hazard to plant and human health. Arsenic exposure generally induces the production of ROS causing progressive oxidative damage and ultimately cell death that can lead to the production of antioxidants. Proline alleviates the damaging effect of oxidative stress in plants by the up-regulation of the antioxidant defense system. In this study, we investigated the protective effects of exogenously applied proline on cell growth, ROS production, glutathione content, and activities of different antioxidant enzymes in cultured tobacco BY-2 cells exposed to arsenate (AsO_4^-) stress. This study describes the molecular mechanisms of lower proline in BY-2 cells under arsenate stress. Previous evidences suggest that arsenic exposure induces the generation of ROS. Our results indicate that arsenate stress increased the ROS levels compared with control and in the presence of arsenate, 0.05 mM and 0.1 mM proline decreased the intracellular ROS level compared with arsenate stress. The mechanisms of how proline mitigates stress in plants are not fully understood but appear to involve its chemical properties and effects on redox systems such as the glutathione pool. Glutathione is the major source of non-protein thiols in plant cells and functions as a key component of the antioxidant network. The increased level of glutathione pool is generally regarded as a protective response against oxidative stress. The reduced glutathione (GSH) content is associated with the protection to oxidative stress in plants. In the presence of AsO_4^-, 0.05 mM proline did not show any effect on total glutathione (Total GSH) content compared with arsenate stress. Furthermore, 0.05 mM proline decreased the GSH and increased the

oxidized glutathione (GSSG) contents compared with arsenate stress. These results suggest that during mitigation process proline maintains glutathione homeostasis by decreasing GSH and increasing GSSG. Arsenate is readily reduced to arsenite through arsenate reductase using GSH as a reductant. In this study, at AsO_4^- stress condition, compared with control, 0.05 mM proline did not show any effects on arsenate reductase activity. Moreover, compared with arsenate stress, 0.05 mM proline significantly increased the arsenate reductase activity. These results suggest that proline mitigates arsenate stress by increasing the arsenate reductase activity which accelerates the conversion of arsenate to arsenite, leading detoxification and sequestration of arsenite. In this study, we also found that the SOD activity is increased at 60 μM arsenic stress compared with control. In the presence of arsenate, proline at 0.05 mM did not show any effect on the SOD activity compared with arsenate stress. The above data indicate that 0.05 mM proline in most of the parameters positively regulated, and recovered the stressing effects of arsenate. Therefore, the mechanism of lower proline-induced stress mitigation is consistent with the previous reports, which is important to understand the mitigatory roles of proline in BY-2 cells.

3.2 INTRODUCTION

Arsenic is an environmental toxin that is found naturally in the environment. The main two forms of inorganic arsenic, arsenate and arsenite, are easily taken up by plant cell. In aerobic environments, arsenic occurs mostly in its oxidized form, arsenate, and has been reported to be taken up by plants via the phosphate transport system (Asher and Reay, 1979; Lee, 1982; Ullrich-Eberius et al., 1989; Meharg and Macnair, 1991, 1992; Meharg and Hartley-Whitaker, 2002; Wang et al., 2002). Once taken up by the plant, arsenate can be readily converted to arsenite, the more toxic of the two forms. Arsenic, when not detoxified, may trigger a sequence of reactions leading to growth inhibition, and stimulation of secondary metabolism, and causes membrane degradation and cell death (Meharg and Hartley

Whitaker, 2002).

Arsenic is also known to induce oxidative stress directly by generating ROS during conversion of its valence forms by inactivating antioxidant molecules through binding with their sulfhydryl (-SH) groups (Sharma, 2012). Arsenic interferes with various metabolic processes and thereby adversely affects the plant metabolism and leads to reduced plant productivity.

In response to different stresses, plants accumulate large quantities of different types of compatible solutes (Serraj and Sinclair, 2002). Proline is a well-known compatible solute that accumulate under metal stress has been correlated with stress tolerance in plants. Proline play significant functions under metal stress and it has been reported that proline accumulation may be a part of stress signal influencing adaptive responses.

Both glutathione (Ogawa, 2005) and proline (Lehmann et al., 2010) perform multiple functions in plants and originated from a common precursor L-glutamate (Moat et al., 2003). Glutathione is a key component of the antioxidant network that functions as a major intracellular antioxidant inside the cell (Cobbett, 2000). It is reported that glutathione can function as an intracellular signaling agent and responsive to changes in the extracellular environment (Fernandez et al., 1997). Previous reports suggest that arsenite form complexes with thiol compounds in plants, due to its high affinity with sulfhydryl groups (Raab et al., 2005). The increased level of glutathione synthesis is considered a means of metal binding capacity as well as a way to increased cellular defense against oxidative stress.

The reduction of arsenate to arsenite is a key step in the arsenic detoxification pathways. Arsenate is readily reduced to arsenite through arsenate reductase using reduced glutathione (GSH) as reductant (Duan et al*.,* 2005; Mukhopadhyay et al., 2000). Though the reduction mechanism is not well understood in plants, the reduction of arsenate to arsenite occurs in a number of plant species (Pickering et al*.,* 2000; Meharg and Hartley-Whitaker, 2002; Salt et al., 2002; Quaghebeur and Rengel, 2003).

Under environmental stresses, plants often produce ROS such as superoxide, hydrogen peroxide and hydroxyl radicals, causing damage to DNA, proteins and lipids. It is evident that arsenic exposure leads to the generation of ROS through the conversion of arsenate to arsenite, a process that rapidly occurs in plants (Flora, 1999; Mascher et al., 2002). To minimize the harmful effects of ROS, plants have evolved an effective scavenging system composed of antioxidant molecules and antioxidant enzymes (Meharg, 1994). However, in some instances this defensive mechanism becomes inadequate to withstand against stressed conditions (Chandrakar et al., 2016).

Report suggest that during stress, proline decreased the denaturation of enzyme, hence can be applied against arsenic stress which readily binds with thiol groups and inactivates enzymes (Chandrakar et al., 2016). Therefore, research on the protective role of proline is a matter of scientific interest for its application against arsenic-stress.

3.3 MATERIALS AND METHODS

3.3.1 *Culture of tobacco BY-2 cells*

Suspension-cultured cells of tobacco (*Nicotiana tabacum* L., cv. BY-2) were used for the arsenic-unadapted cell lines (Murata et al., 1994a, b). The modified LS medium (Linsmaier and Skoog, 1965) was used as a standard medium. The BY-2 cells were cultured and maintained as described previously (Murata et al., 1994a, b). The culture and maintenance of cells were described in Chapter 2.

3.3.2 *Estimation of proline content*

Proline was measured following the method of Bates et al. (1973). Aliquots of BY-2 cells were washed with 0.2 M sorbitol. Cells were ground and homogenized using liquid N_2 in 10 mL of 3% aqueous sulfosalicylic acid. The homogenate was centrifuged at 12,000 *g* for 15 min at

4^0C. Two mL of the supernatant were reacted with 2 mL of acid ninhydrin (1.25 g ninhydrin dissolved in 30 mL of glacial acetic acid and 20 mL 6M phosphoric acid) and 2 mL of glacial acetic acid for 1 h at 100^0C, and the reaction was then terminated in an ice bath. The colored reaction mixture was extracted with 4 mL of toluene and the absorbance was recorded at 520 nm. The proline content was obtained from the standard curve.

3.3.3 *Isolation of genomic DNA and gel electrophoresis*

Approximately 200 mg of fresh BY-2 cells was ground and homogenized with a mortar and pestle using liquid nitrogen in 500 µL of extraction buffer containing 3% cetyltrimethylammonium bromide, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, and 0.2 % (v/v) *β*-mercaptoethanol. The mixture was incubated at 60 ^{0}C for 45 min. After centrifugation at 11,000 *g* for 10 min at room temperature, the supernatant was transferred to a new microtube and then mixed well with an equal volume of chloroform. After centrifugation at 11,000 *g* for 10 min at room temperature, the aqueous upper phase was collected. The DNA was precipitated with an equal volume of isopropanol, washed in 70% (v/) ethanol, dried, and suspended in sterile distilled water. The DNA was then electrophoresed on a 1% (w/v) agarose gel followed by visualization with ethidium bromide.

3.3.4 *Detection of ROS*

ROS in arsenate stressed BY-2 cells was measured using 2´, 7´-dichlorodihydroflurescein diacetate (H_2DCF-DA) as described previously (Biswas and Mano, 2015). BY-2 cells were collected and washed with distilled water by centrifugation. Cells were then incubated in 20 µM H_2DCF diacetate in PBS at 37 ^{0}C for 30 min and washed two times with PBS. The fluorescence was monitored using a fluorescence microscope.

3.3.5 *Measurement of glutathione content*

GSH contents were determined according to Baker et al. (1990) method. An aliquot of BY-2 cells was ground using liquid nitrogen (N_2) and homogenized with extraction buffer (50 mM KH_2PO_4, 5 mM EDTA, pH 8.0). The homogenate was centrifuged at 9500*g* for 10 min followed by deproteinization with sulphosalicylic acid (4%) and the supernatant was collected. In addition, for the measurement of GSSG content, 2-vinylpyridine (97%) and triethanolamine (20%) were added to the supernatant. Then 50 µL of the supernatant was taken in the wells and 100 µL of the reaction mixture (NADPH, DTNB, KH_2PO_4 and glutathione reductase) was added. The absorbance was recorded at 412 nm using a microplate reader (Nippon Bio-Rad Laboratories (680), Tokyo, Japan). The GSH content was calculated by subtracting the GSSG from the total GSH.

3.3.6 *Extractions and measurements of arsenate reductase*

For preparation of sample solution, approximately 0.5 g of fresh BY-2 cells was ground with a mortar and pestle that were chilled with liquid nitrogen and homogenized in 3 ml buffer solution containing 50 mM MOPS ((3-(N-morpholino) propane sulfonic acid) and 50 mM MES((2-(N-morpholino) ethane sulfonic acid), which was adjusted to pH of 6.5 with 1 M NaOH. The homogenate was centrifuged at 10,000 × *g* for 30 min at 4 ^{0}C. The supernatant was filtered through Qualitative Advantec 2 filter paper. The resulting filtrate was collected and passed through Sephadex PD-10 desalting columns (Duan et al., 2005). The final filtrate was the extract containing arsenate reductase. All the above steps were carried out on ice.

Arsenate reductase activity was assayed using the methods described by Shi et al. (1999) and Duan et al. (2005). The assay buffer was 50 mM MOPS and 50 mM MES, pH 6.5, with a total volume 1.5 mL containing 1.0 mM NADPH, 1 unit yeast glutathione reductase, 1 mM GSH, 10 mM sodium arsenate. The extract (100 µL) was pre-incubated at 30 ^{0}C for 5 min in a buffer containing yeast glutathione reductase and GSH. Small volumes of NADPH

and sodium arsenate were then added and mixed thoroughly to start the reaction. All steps were carried out at 30 ^{0}C. Arsenate reductase activity was monitored by the decrease in NADPH absorbance at 340 nm and the NADPH oxidation was calculated using a molar extinction coefficient of 6200 for NADPH at 430 nm.

3.3.7 *Extractions and measurements of superoxide dismutase*

Approximately 200 mg of fresh BY-2 cells was harvested and ground with a mortar and pestle that were chilled with liquid nitrogen and added 500 µL of phosphate buffer solution (pH 8.0). SOD activity was measured by using SOD Test Kit (Wako). For each SOD activity measurement, 100 µL of the sample solution was taken in microtube for sample (S) and sample. blank (S.BL). Hundred µL of distilled water was taken for blank (BL) and blank. blank (BL.BL). After that 1.0 mL of coloring reagent was added to each tube and 1.0 mL of enzyme solution was added for sample (S) and blank (BL). One mL of blank reagent was added for sample. blank (S.BL) and blank. blank (BL.BL). After incubation at 37^{0}C for 20 min, 2.0 mL reaction stopper was added to each tube. The absorbance was measured at 560 nm. The activity was calculated (Inhibition rate %) using the following equation:

$$\text{SOD activity (Inhibition \%)} = \{(E_{BL}-E_{BL\cdot BL}) - (E_S-E_{S\cdot BL})/ (E_{BL}-E_{BL\cdot BL})\} \times 100$$

3.3.8 *Measurement of protein*

Protein determinations were carried out using the method of Bradford (1976) with BSA as standard.

3.3.9 *Statistical analysis*

Unless stated otherwise, the significance of differences between the mean values of all parameters was assessed by Tukey's test. Differences at the level of $p \leq 0.05$ were considered as significant.

3.4 RESULTS

3.4.1 *Proline content in response to application of exogenous proline*

To investigate whether exogenously applied proline induces the accumulation of proline content under AsO_4^- stress, we measured the intracellular proline contents in BY-2 cells both at arsenate stress and non-stress condition (Fig. 3.1). The proline content was significantly increased at 60 μM AsO_4^- compared with control, showing that AsO_4^- stress induced the accumulation of proline. Exogenously applied 0.05 mM proline showed remarkable increase in the accumulation of proline compared with control. In the presence of arsenate, 0.05 mM proline led to significant increase in proline content which is similar with 60 μM AsO_4^- stress. Proline content was significantly increased at 0.1 mM both at AsO_4^- stress and non-stress condition. These results suggest that both under As-stressed and non-stressed conditions, proline content was increased in a concentration-dependent manner in response to the application of exogenous proline.

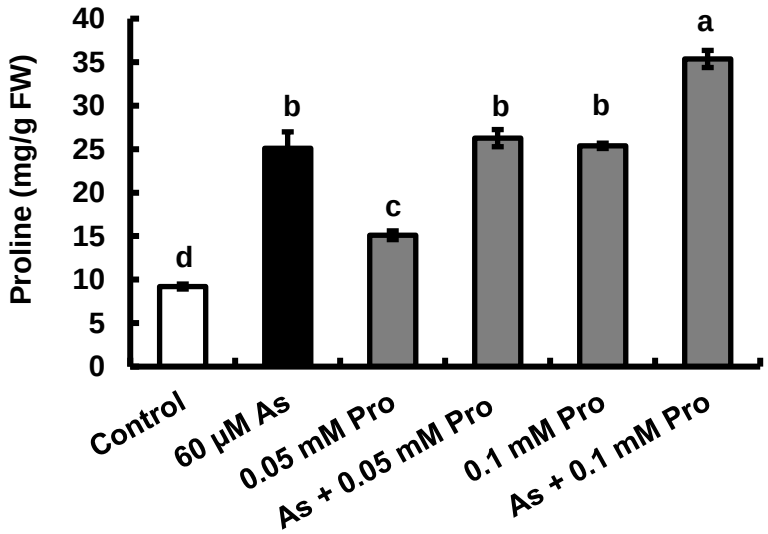


Figure 3.1 Proline content in response to exogenous proline application. The Proline content was increased both under 60 μM AsO_4^--stressed and non-stressed conditions in a concentration-dependent manner in response to the application of exogenous proline. Averages of proline contents from three independent experiments (n = 3) are shown.

3.4.2 *DNA fragmentation*

To investigate whether DNA fragmentation occurred in BY-2 cells under arsenate stress, DNA was extracted from BY-2 cells and subjected to electrophoresis on agarose gel prior to staining with ethidium bromide. No DNA fragmentation was detected in the arsenate stressed cells irrespective of the presence or absence of proline (Fig. 3.2).

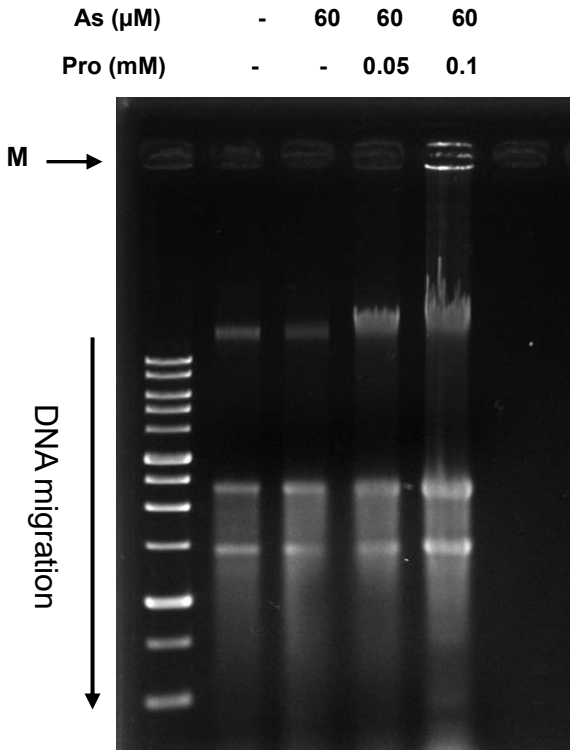


Figure 3.2 DNA fragmentation in arsenate (As) stress BY-2 cells in response to exogenous proline (Pro) application. DNA was extracted and subjected to electrophoresis on 1 % agarose gel. Photographs shown are representative of four independent experiments. M, 1 kb DNA marker.

3.4.3 *Intracellular ROS level*

We investigated whether proline could inhibit arsenate-induced ROS accumulation in BY-2 cells (Fig. 3.3). Arsenate stress resulted in a significant increase in accumulation of intracellular ROS in BY-2 cells compared with control. In the presence of arsenate, proline at 0.05 mM and 0.1 mM significantly inhibited the arsenate induced ROS accumulation in BY-2 cells compared with arsenate stress (Fig. 3.3).

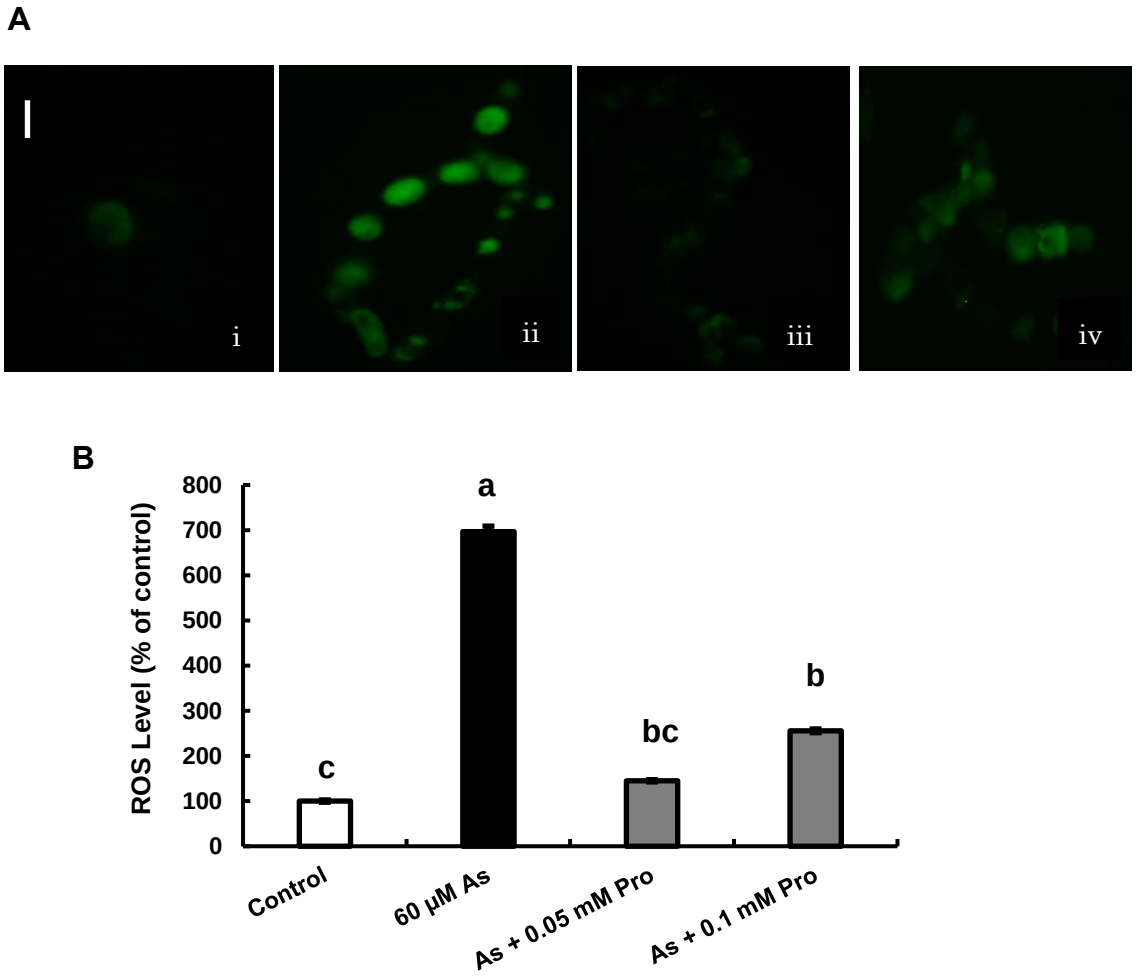
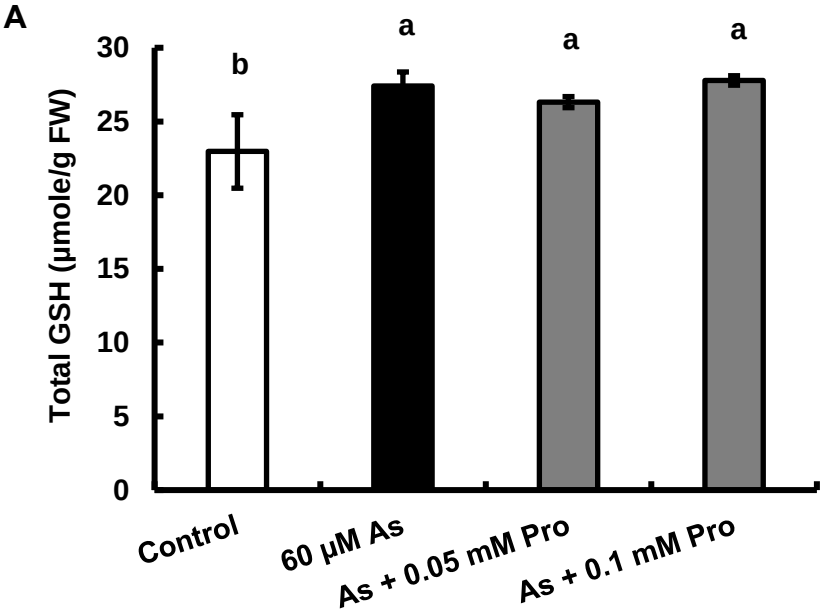


Figure 3.3 Intracellular ROS levels in tobacco BY-2 suspension cells under arsenate (As) stress in the presence or absence of proline (Pro). Four-day-cultured cells were incubated in As stress media with different concentration of Pro and DCF fluorescence was recorded using fluorescence microscope. A, Typical photographs are shown: untreated cells as a control (i), 60 μM As (ii), As + 0.05 mM Pro (iii), and As + 0.1 mM Pro (iv). Bar = 10 μm. B,

DCF fluorescence intensity of BY-2 cells. The fluorescence intensity is shown as a ROS level. Values represent the mean ±SE from four independent experiments. Bars with same letters are not significantly different at $P \leq 0.05$.

3.4.4 *Effects of exogenous proline on glutathione content at 60 µM arsenate- stressed BY-2 cells*

We examined the effects of proline on the total GSH, GSSG and GSH contents in BY-2 cells at 60 µM (Fig. 3.4) arsenate stress. Arsenate stress significantly increased the total GSH content in BY-2 cells (Fig. 3.4A) compared with control. In this study, exogenous proline at 0.05 mM did not show any effect on total GSH content compared with AsO_4^- stress. In the presence of arsenate, proline at 0.05 mM decreased the GSH content and increased the GSSG content compared with arsenate stress, whereas 0.1 mM proline did not show any effect on total GSH, GSH and GSSG contents compared with arsenate stress (Fig. 3.4). These results indicated that proline at 0.05 mM mitigates arsenate stress in BY-2 cells by decreasing GSH and increasing GSSG contents.



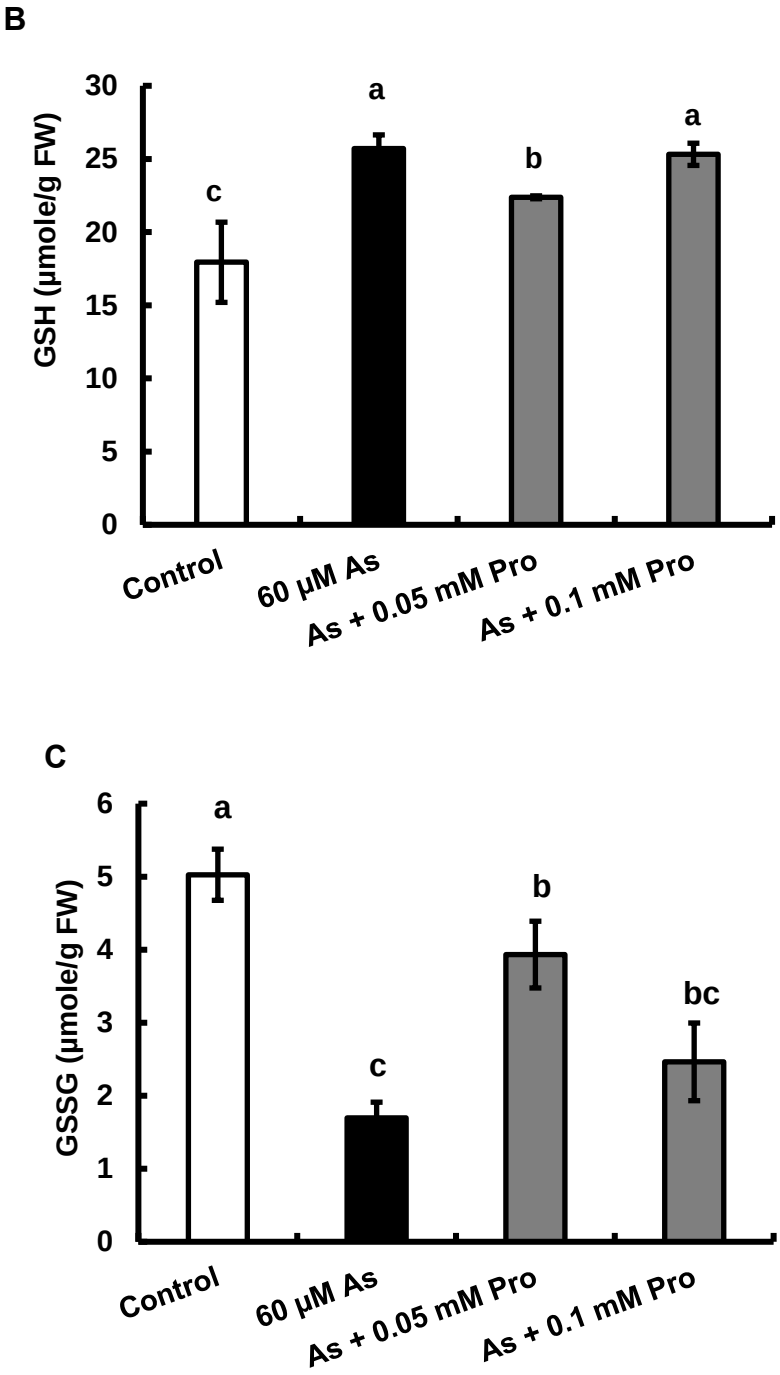


Figure 3.4 Glutathione content at 60 µM AsO_4^--stressed BY-2 cells in presence of lower concentration of exogenous proline (Pro). Total GSH (A), reduced glutathione (GSH) (B), and oxidized glutathione (GSSG) (C) content in As-unadapted tobacco BY-2 cells induced by Pro under AsO_4^-stress. Values represent the mean ±SD (n=3).

3.4.5 *Effects of exogenous proline on the activity of arsenate reductase in AsO_4^- stressed BY-2 cells*

Fig. 3.5 shows effect of proline on the activities of arsenate reductase (AR) in tobacco BY-2 cells under AsO_4^- stress. In the presence of 60 μM AsO_4^-, compared with control, 0.05 mM proline did not show any effects on AR activity. However, compared with arsenate stress, 0.05 mM and 0.1 mM proline significantly increased the arsenate reductase activity. These results suggest that lower level of proline mitigates arsenate stress by increasing the AR activity which accelerates the conversion of arsenate to arsenite.

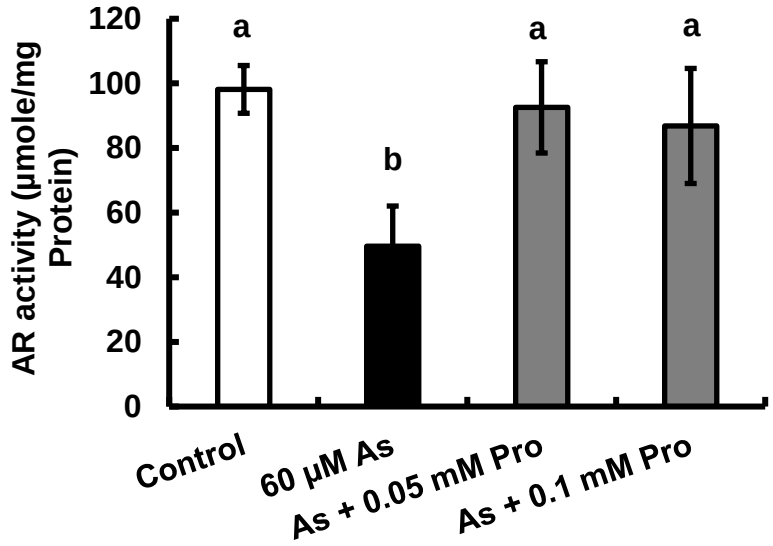


Figure 3.5 Arsenate reductase (AR) activity of arsenate unadapted tobacco BY-2 suspension cells induced by proline under AsO_4^- stress. Four-day-cultured cells were co-treated with 0.05 mM and 0.1 mM exogenous proline respectively at 60 μM AsO_4^- stress medium. Means ± SD of three independent experiments are shown.

3.4.6 *Superoxide dismutase activity*

Arsenate stress significantly increased the superoxide dismutase (SOD) activity in tobacco BY-2 cells represented in Fig. 3.6. The SOD activity in control condition is lower than the AsO_4^- stress. In this study, proline at 0.05 mM and 0.1 mM did not show any effect on the SOD activity in the presence of AsO_4^-.

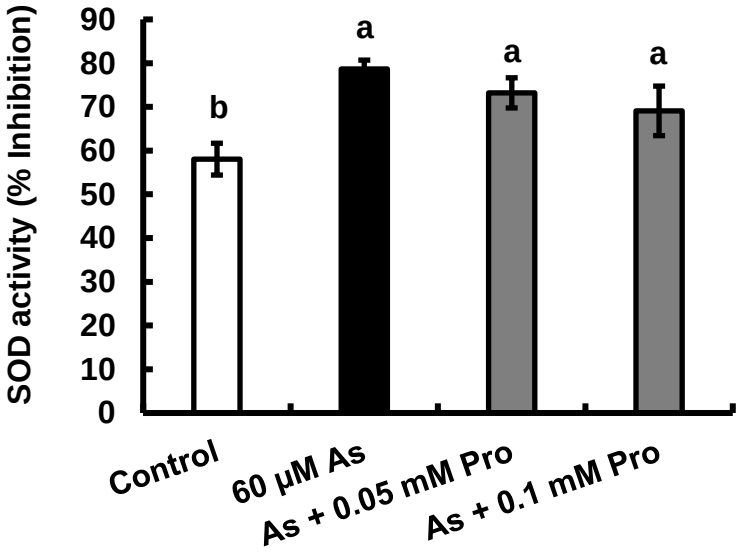


Figure 3.6 Superoxide dismutase (SOD) activity of arsenate-unadapted tobacco BY-2 suspension cells induced by lower concentration of proline (Pro) under AsO_4^- stress. Suspension cells of a 7-day-old culture were inoculated into different fresh media, and cells were collected at 4-days after inoculation. Values represent the mean ±SD (n=3).

3.5 DISCUSSION

We have investigated the effect of exogenous proline on arsenate stressed BY-2 cells. We found that proline at lower concentration mitigate AsO_4^- -induced growth inhibition by suppressing Evans Blue stained cells and increasing the total number of cell. It was reported that proline ameliorates heavy-metal toxicity in plants. However, the mechanisms of how proline mitigates arsenate stress in plants are not fully understood but appear to involve its chemical properties and effects on redox systems such as the glutathione pool and the activities of antioxidant enzymes. Our recent study has indicated that exogenous proline increased the intracellular accumulation of proline in a concentration dependent manner as well as proline increased the inhibition of ROS production at arsenate stress. In our study, the lower proline decreased the reduced glutathione and increased the oxidized glutathione contents compared with arsenate stress. We also found that in the presence of arsenate, exogenous proline increased the activity of arsenate reductase, and thus we elucidate the role of lower concentration of proline in BY-2 cells under arsenate stress.

There are significant evidences that arsenic exposure induces the generation of ROS during the conversion of arsenate to arsenite (Hartley-Whitaker et al., 2001). Our results indicate that arsenate stress increased the ROS levels compared with control. The present data showed that in the presence of arsenate 0.05 mM and 0.1 mM proline decreased the intracellular ROS level compared with arsenate stress (Fig. 3.3B). These results suggest that lower proline decreased arsenate toxicity by increasing the inhibition of ROS production.

A non-enzymatic antioxidant, GSH, constitutes an important plant defense system against environmental stresses including heavy metals (Wingate et al., 1988). It was reported that glutathione and glutathione metabolism is the primary detoxification strategy by which plants tolerate arsenate stress (Mishra et al., 2008). Previous studies suggested that the GSH content is either increase or decrease or maintains homeostasis in response to mitigate stress-induced changes in plants and it was also reported that glutathione content is

significantly increased in plants upon arsenic exposure (Srivastava et. al., 2007). Our data also showed that the total GSH content is increased in AsO_4^--stressed BY-2 cells (Fig.3.4A), which indicates that glutathione is an important element that regulates stress-induced changes in plants. In this study, we also found that exogenous proline at 0.05 mM decreased the GSH and increased the GSSG contents compared with arsenate stress (3.4B and C). It was reported that the detoxification of heavy metals by GSH results in the rapid depletion of GSH in the cytoplasm (Polhuijs et al., 1992). On the other hand, Agarwal et al. (2011) reported that GSH content is increased in proline-treated bean (*Phaseolus vulgaris*) plants that mitigate selenium stress. Though it seems contradictory according to our findings but the response of GSH and GSSG levels in terms of mitigation of stress is similar with the previous one. It is well established that the detoxification of As and Cd requires GSH and PCs in plants (Howden et al., 1995; Shi et al., 1996; Cobbet et al., 1998; Dhankher et al., 2002; Verbruggen *et al.,* 2009). Agarwal (2011) reported that selenium stress is mitigated by increasing GSH level as well as we found that during mitigation process at 0.05 mM proline maintains glutathione homeostasis by decreasing GSH content and increasing the GSSG content.

Arsenic is detoxified by complexation with the thiol-rich peptide such as GSH. The GSH is required for the reduction of arsenate to arsenite by arsenate reductase (Liu and Rosen, 1997) and mitigates arsenic stress. Although arsenate can be reduced nonenzymatically in the presence of GSH (Delnomdedieu et al., 1994), this process is too slow to be significant biologically. In this study, compare with control, 0.05 mM proline did not show any effects on arsenate reductase activity. Moreover, exogenous proline at 0.05 mM significantly increased the arsenate reductase activity compared with arsenate stress (Fig. 3.5). These results suggest that proline mitigates arsenate stress by increasing the arsenate reductase activity which accelerates the conversion of arsenate to arsenite, leading detoxification and sequestration of arsenite.

The activity of antioxidant enzymes is important for stress mitigation. It is reported that arsenic exposure induces the production of ROS that can lead to the production of antioxidant metabolites and numerous enzymes involved in antioxidant defense mechanisms (Finnegan and Chen, 2012). Reports suggest that antioxidant enzyme such as superoxide dismutase and peroxidases plays prominent role in response to arsenate stress and the activity of SOD is increased at AsO_4^- stress condition (Armendariz et al., 2016). Our data also showed that the SOD activity is increase at arsenate stress condition (Fig. 3.6), which indicates that SOD play an important role that regulates stress-induced changes in plants. Our previous reports have shown that proline increased antioxidant defense systems and improve salt tolerance and Cd tolerance in BY-2 cells (Okuma et al., 2004; Hoque et al., 2007; Banu et al., 2009). On the other hand, Salim et al. (2009) did not found increased level of SOD activity in BY-2 cell under Cd stress. In our study, in the presence of arsenate, proline at 0.05 mM and 0.1 mM did not show any effect on the SOD activity compared with arsenate stress (Fig. 3.6). Report also suggests that proline alleviates Cd toxicity in *Solanum nigrum* by detoxifying ROS and increasing the activity of SOD (Xu et al., 2009). The above reports with our findings suggest that the mitigatory role of proline might depend on some conditions such as type of stress, and plant species.

However, the physiological bases of arsenic metabolism remain unclear and previous reports indicate that proline is involved in plant heavy metal stress tolerance by different mechanisms such as osmo and redox regulation, metal chelation and scavenging of radicals. To combat arsenic stress, plants modulate a number of pathways that operate to keep the cellular concentration of metal ion to a minimum level via thiol-mediated complexation (Bleeker et al., 2006). In conclusion, based on reverse and forward studies, proline at lower concentration decreased the production of ROS and ensures glutathione turnover during arsenate stress as a tolerance mechanism in arsenate stressed tobacco BY-2 cells.

CHAPTER 4

Comparison between the effects of proline and the effects of other osmolytes in BY-2 cells under arsenate stress

4.1 ABSTRACT

Arsenic is the most toxic metalloid widely concerned and widely distributed in the environment. Plants respond to arsenic toxicity by a variety of mechanisms and when not detoxified, may trigger a sequence of reactions leading to growth inhibition, and stimulation of secondary metabolism. The report suggests that the accumulation of proline may be a part of stress signal influencing adaptive responses. In this study, we investigated the effects of exogenously applied proline on cell growth, glutathione content, and activities of different antioxidant enzymes in cultured tobacco BY-2 cells exposed to arsenate (AsO_4^-) stress. Here, we also examine the effects of some stress-related organic molecules on the BY-2 cell growth both under AsO_4^- stress and non-stress conditions to compare the results with proline effects, as well as to clarify the proline-enhanced cell growth reduction by arsenate. In the presence of arsenate, 0.5 mM and 1 mM proline did not show any effect on total GSH and GSH and GSSG contents but 10 mM proline decreased it compared with arsenate stress. Moreover, proline at 10 mM significantly decreased the arsenate reductase activity as well as SOD activity. It has been reported that there is a considerable interconnection between proline and arginine catabolic pathways when applied exogenously. Arginine is degraded to proline and then further catabolized following the metabolic pathway similar to proline. Here, we found that 10 mM arginine did not affect BY-2 cell growth, but in the presence of arsenate, like 10 mM proline, 10 mM arginine enhanced the cell growth reduction by arsenate. Alanine, unlike proline, follows separate catabolic pathway and report suggests that alanine removes the feedback inhibition of proline biosynthesis pathway and enhances the accumulation of

proline during stress condition. Here, exogenous alanine did not affect BY-2 cell growth at 0.5 mM to 10 mM. In the presence of AsO_4^-, neither 0.5 mM nor 1 mM alanine affected the cell growth but 10 mM alanine significantly recovered the AsO_4^--induced growth inhibition. These results suggest that exogenous alanine mitigates arsenate stress and as alanine, does not follow catabolic pathway similar to proline, arsenate did not show any inhibitory actions. Glutamate is the precursor of proline biosynthesis in plants. During stress condition, proline is synthesized from glutamate that functions as a protective molecule. In the present study, glutamate at 0.5 mM to 10 mM did not show any effect on BY-2 cell growth and did not increase arsenate induced cell growth reduction. Glycine betaine is a compatible solute that accumulates in plants and mitigates stresses. Here, I found that exogenous betaine at 0.5 mM and 1 mM did not affect BY-2 cell growth and did not improve AsO_4^--induced cell growth reduction. Unlike proline effects, the arsenate in the presence of glutamate and betaine did not enhance the cell growth reduction, suggesting that arsenate, particularly in the presence of proline, increased the sensitivity of BY-2 cells. In conclusion, exogenous proline plays dynamic roles in BY-2 cells under arsenic stress, which depends on its level of concentration. Together, our results suggest that exogenous proline at higher concentration does not alleviate arsenate toxicity but enhances the sensitivity of BY-2 cells to arsenate.

4.2 INTRODUCTION

Arsenic a toxic substance released into the environment, contributing to a variety of toxic effects on living organisms. Arsenic toxicity has become one important constraint to crop productivity and quality. Among inorganic and organic species of arsenic, arsenite is the more toxic form due to its propensity to bind to sulfhydryl groups, which causes detrimental effects on general protein functioning (Tripathi et al., 2007).

Glutathione, a non-enzymatic antioxidant, is a low molecular weight thiol implicated in a wide range of metabolic processes and constitutes an important plant defense systems

against environmental stress including heavy metal stress (Hossain et al., 2010, 2011). It is one of the major antioxidant material in plants that found abundantly in all cell compartments. It is reported that glutathione and glutathione dependent enzymes play an important role in detoxification of heavy metals (Hossain et al., 2012) such as arsenic. Glutathione acts as a thiolate donor in plant heavy metal stress (Pickering et al., 2000). An increased level of GSH concentration during heavy metal stress is always considered as a protective way in plants.

The reduction of arsenate to arsenite is regarded as another aspect of arsenic detoxification in plants (Zhang et al., 2002) that required GSH as a reductant. Arsenate can also be reduced by GSH alone but the process is usually very slow and incomplete, whereas the enzymatic reduction under optimum condition was much faster. Arsenate reductase is a specific catalyzer to reduce arsenate to arsenite in plants (Mukhopadhyay et al., 2000; Duan et al., 2005).

Under stress condition, plant produced ROS and within a cell, the SODs constitute the first line of defense against ROS (Alscher et al., 2002). To avoid the damages effectively, the anti-oxidative enzyme system would be activated. Several studies reported that these enzymes (e.g. superoxide dismutase, SOD) are important to defend the plant oxidative stress caused by arsenate (Cao et al., 2004; Srivastava et al., 2005).

Amino acids are the building blocks of proteins and are known to be induced significantly upon heavy metal exposure (Davies et al.,1987). Amino acids and their derivatives have been reported to chelate metal ions, thus conferring metal tolerance to plants. Plants have evolved a variety of adaptive mechanisms to respond to heavy metal stress including arsenic stress. It is reported that accumulation of compatible solutes is the main adaptive mechanism in metal stress situation (Shah and Dubey, 1998; Mehta and Gaur, 1999; Sharma and Dietz, 2006; Ashraf and Foolad, 2007). When exposed to stressful conditions, plants accumulate an array of metabolites, particularly amino acids. Proline, an amino acid, plays a highly beneficial role in plants exposed to various stress conditions

(Hayat et al., 2012). Proline is the most common compatible solutes that occur in a wide variety of plants.

Arginine is one of the most versatile amino acids and it has multiple metabolic fates. In addition to serving as a constituent of proteins, arginine is a precursor for biosynthesis of polyamines, urea, and proline as well as the cell signaling molecules glutamine and nitric oxide (Chen et al., 2004; Liu et al., 2006). The report suggests that Arginine follows proline degradation pathway when applied exogenously (Thompson, 1980). Alanine, a nonessential amino acid and the accumulation of alanine are increased at several stress conditions in plants. Glycine betaine or betaine, a quaternary ammonium compound, is regarded as one of the most effective osmoprotectants in plants, bacteria and algae (Delauney and Verma, 1993). Among the compatible solutes, betaine is a particularly effective protectant against abiotic stress (Sakamoto and Murata, 2000, 2001, 2002).

However, the molecular mechanism by which proline, arginine, alanine, glutamate and betaine regulated stress-related signals in plants remain to be investigated.

4.3 MATERIALS AND METHODS

4.3.1 *Culture of tobacco BY-2 cells*

Suspension-cultured cells of tobacco (*Nicotiana tabacum* L., cv. BY-2) were used for the arsenic-unadapted cell lines (Murata et al., 1994a, b). The modified LS medium (Linsmaier and Skoog, 1965) was used as a standard medium. The 0.5 mM, 1 mM and 10 mM proline, and arginine media were the 40 μM as well as 60 μM AsO_4^- media and containing 0.5 mM, 1 mM and 10 mM proline and arginine respectively. The 0.5 mM, 1 mM and 10 mM alanine, and glutamate media were the 60 μM AsO_4^- media containing 0.5 mM, 1 mM and 10 mM alanine and glutamate respectively. The 0.5 mM and 1 mM betaine media were the 60 μM AsO_4^- media containing 0.5 mM, and 1 mM betaine respectively. The BY-2 cells were cultured

and maintained as described previously (Murata et al., 1994a, b). The culture and maintenance of cells were described in Chapter 2.

4.3.2 *Measurement of BY-2 cell growth*

The growth of BY-2 cells was measured as described previously (Murata et al., 1994a, b). The measurement procedure of BY-2 cell growth was described in Chapter 2. Cultured cells transferred to the different fresh media were used for the experiments at 4 d after inoculation.

4.3.3 *Estimation of endogenous proline*

Proline was measured following the method of Bates et al. (1973). The detailed of this method was described in Chapter 3. The proline content was obtained from the standard curve.

4.3.4 *Measurement of glutathione content*

GSH contents were determined according to Baker et al. (1990) method. An aliquot of BY-2 cells was ground using liquid nitrogen (N_2) and homogenized with extraction buffer (50 mM KH_2PO_4, 5 mM EDTA, pH 8.0). The homogenate was centrifuged at 9500*g* for 10 min followed by deproteinization with sulphosalicylic acid (4%) and the supernatant was collected. The measurement procedure of the total GSH and GSSG content was described in Chapter 3. The GSH content was calculated by subtracting the GSSG from the total GSH.

4.3.5 *Extractions and measurements of arsenate reductase*

Arsenate reductase activity was assayed using the methods described by Shi et al. (1999), Mukhopadhyay et al. (2000) and Duan et al. (2005). The detailed procedure for the measurement of arsenate reductase activity was described in Chapter 3.

4.3.6 *Extractions and measurements of superoxide dismutase*

Approximately 200 mg of fresh BY-2 cells were harvested and ground with a mortar and pestle that were chilled with liquid nitrogen and added 500 µL of Phosphate buffer solution (pH 8.0). SOD activity was measured by using SOD Test Kit (Wako). The detailed explanation of SOD measurement procedure was described in Chapter 3. The activity was calculated (Inhibition rate %) using the following equation:

SOD activity (Inhibition %)= $\{(E_{BL}-E_{BL\cdot BL}) -(E_S-E_{S\cdot BL})/ (E_{BL}-E_{BL\cdot BL})\}^{x} 100$

4.3.7 *Determination of lipid peroxidation*

Lipid peroxidation was assayed as described by Okuma et al. (2004) and Banu et al., (2008) by measuring the malondialdehyde (MDA) content. An aliquot of BY-2 cells was grounded using liquid Nitrogen (N_2) and homogenized with 2 ml of 5% (w/v) trichloroacetic acid (TCA). After centrifuging at 12,000g for 15 min, the supernatant was diluted with 1.2 ml of 20% (w/v) trichloroacetic acid containing 0.5% (w/v) thiobarbituric acid. The mixture was heated at 95^0C for 25 min and the reaction was stopped by placing the tubes in an ice bath. The samples were centrifuged at 7,500g for 5 min. The absorbance of the supernatant was measured at 532 nm, and the value for non-specific turbidity at 600 nm was subtracted. MDA content was calculated using extinction co-efficient of 155 mM^{-1} cm^{-1}.

4.3.8 *Measurement of protein*

Protein determinations were carried out using the method of Bradford (1976) with BSA as standard.

4.3.9 *Statistical analysis*

Unless stated otherwise, the significance of differences between the mean values of all parameters was assessed by Tukey's test. Differences at the level of $p \leq 0.05$ were considered as significant.

4.4 RESULTS

4.4.1 *Proline content in response to application of exogenous proline*

To examine whether exogenous proline induces the accumulation of proline content under AsO_4^- stress, we measured the intracellular proline contents in BY-2 cells both at arsenate stress and non-stress condition (Fig. 4.1). The proline content was significantly increased at 60 μM AsO_4^- compared with control, showing that AsO_4^- stress induced the accumulation of proline. Exogenously applied 0.5 mM, 1 mM and 10 mM proline showed a remarkable increase in the accumulation of proline compared with control. Proline content was significantly increased at 0.5 mM to 10 mM both at AsO_4^- stress and non-stress condition. These results suggest that endogenous proline content was increased in a concentration-dependent manner in response to the application of exogenous proline.

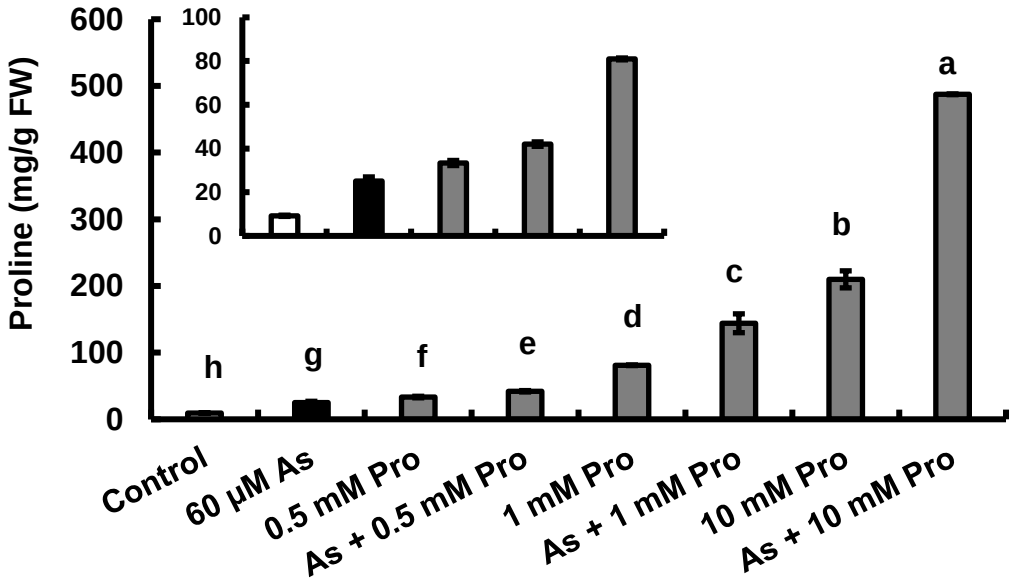
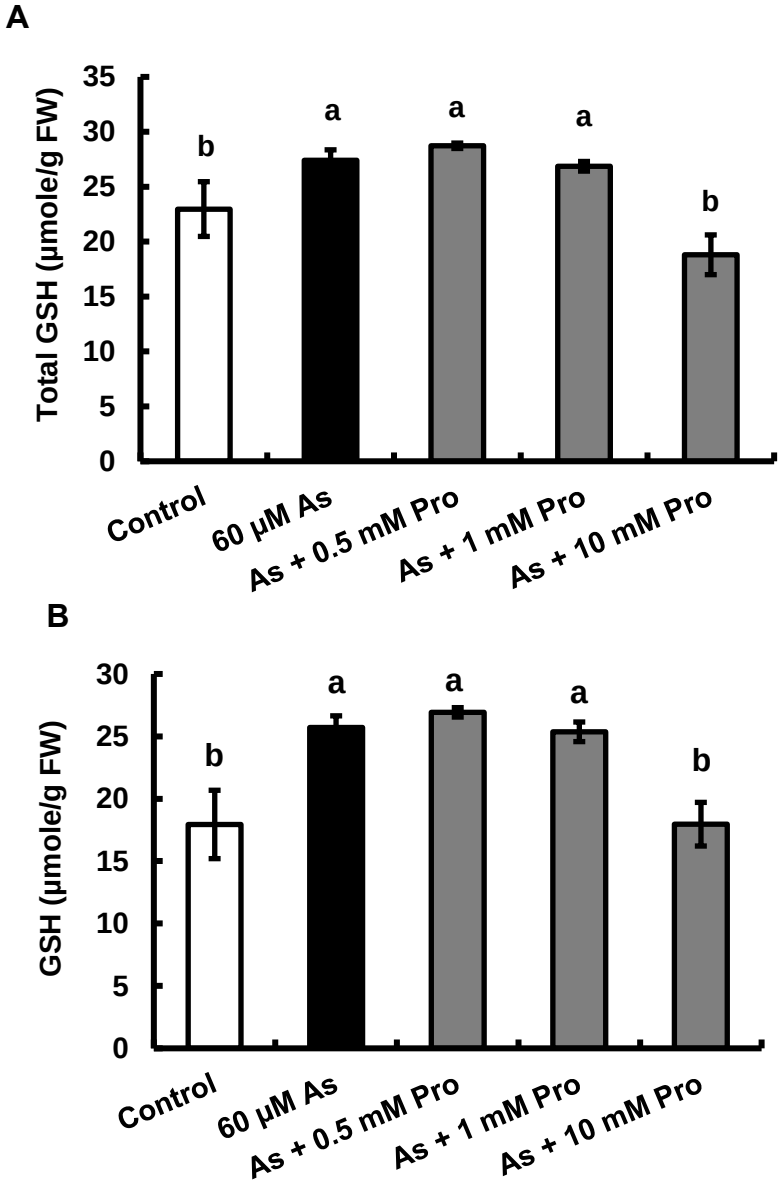


Figure 4.1 Proline (Pro) content in response to exogenous proline application. The proline content was increased both under 60 μM arsenate (As) stressed and non-stressed conditions in a concentration-dependent manner in response to the application of exogenous proline. Averages of proline contents from three independent experiments (n = 3) are shown.

4.4.2 *Effects of exogenous proline on glutathione content at 60 µM arsenate- stressed BY-2 cells*

We examined the effects of proline on the total GSH, GSH and GSSG contents in BY-2 cells at 60 µM (Fig. 4.2) arsenate stress condition. Arsenate stress significantly increased the total GSH content in BY-2 cells (Fig. 4.2A) compared with control. In this presence of arsenate, exogenous proline at 0.5 mM and 1mM did not show any effect on total GSH, GSH and GSSG content whereas, 10 mM proline decreased them compared with arsenate stress (Fig. 4.2A, B and C). These results indicated that proline decreased the glutathione pool and therefore, sensitizes the BY-2 cells to arsenate.



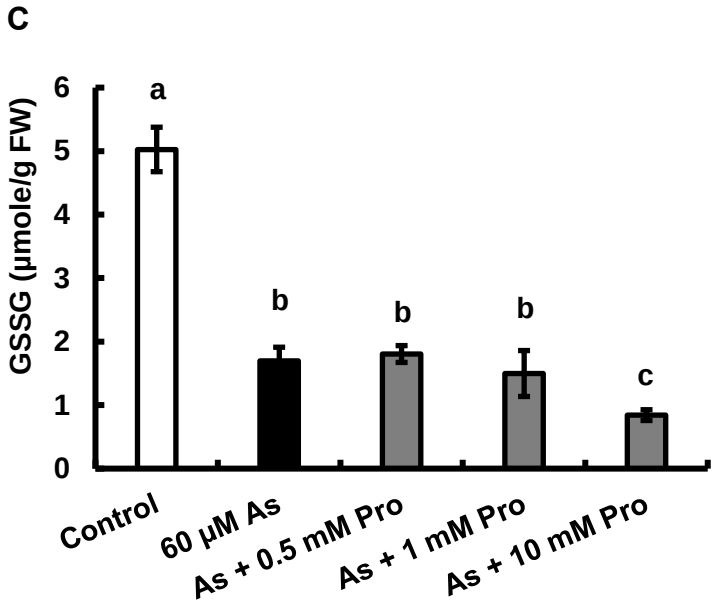
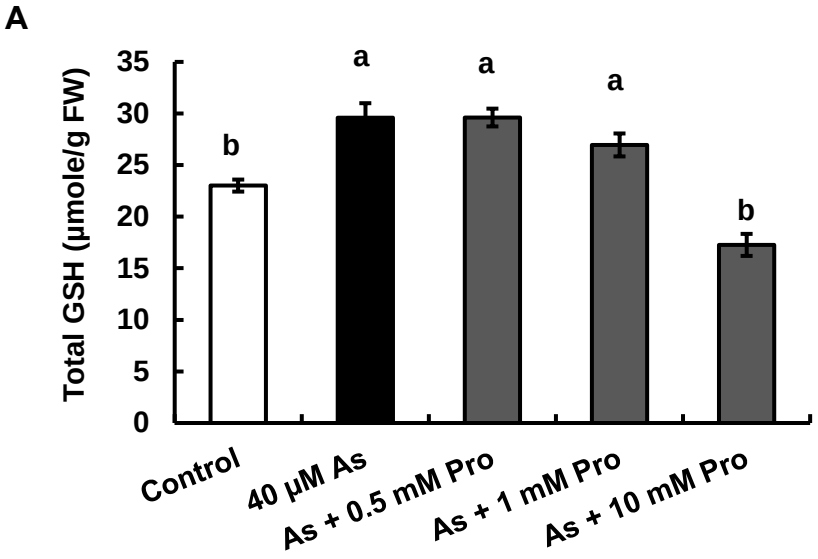
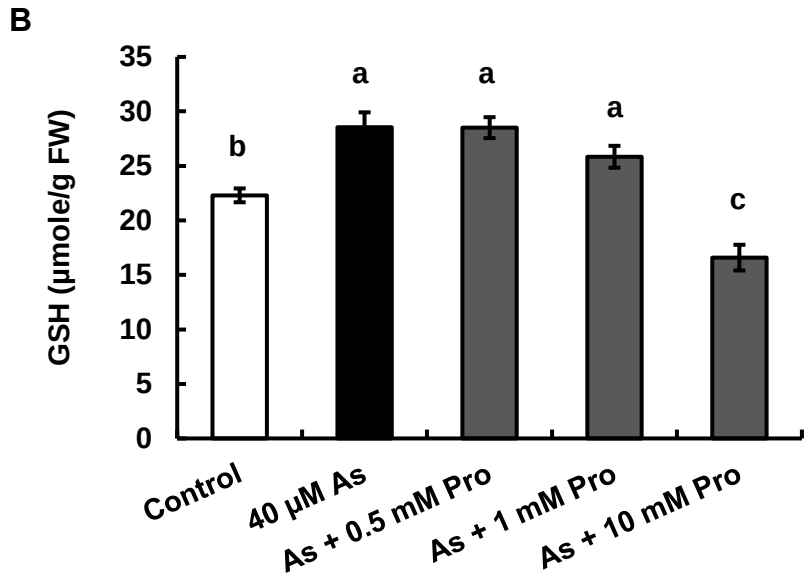


Figure 4.2 Glutathione content at 60 µM arsenic (As)-stressed BY-2 cells in presence of higher concentration of exogenous proline (Pro). Total GSH (A), reduced glutathione (GSH) (B), and oxidized glutathione (GSSG) (C) content in As-unadapted tobacco BY-2 cells induced by Pro under AsO_4^-stress. Values represent the mean ±SD (n=3).

4.4.3 *Effects of exogenous proline on glutathione content at 40 µM arsenate- stressed BY-2 cells*

We investigated the effects of proline on the total GSH, GSH and GSSG contents in BY-2 cells at 40 µM AsO_4^- (Fig. 4.3A, B and C). Arsenate stress significantly increased the total GSH content in BY-2 cells (Fig.4.3A) compared with control. Exogenous application of 0.5 mM and 1 mM proline did not change the contents of total GSH, GSH and GSSG in BY-2 cells treated with arsenate but 10 mM proline significantly decreased them. Results indicate that arsenate inhibits the growth of BY-2 cells by decreasing the glutathione pool in presence of exogenous proline.


A
Total GSH (µmole/g FW)
35
30
25
20
15
10
5
0
a
a
a
b
b
Control
40 µM As
As + 0.5 mM Pro
As + 1 mM Pro
As + 10 mM Pro


B
GSH (µmole/g FW)
35
30
25
20
15
10
5
0
a
a
a
b
c
Control
40 µM As
As + 0.5 mM Pro
As + 1 mM Pro
As + 10 mM Pro

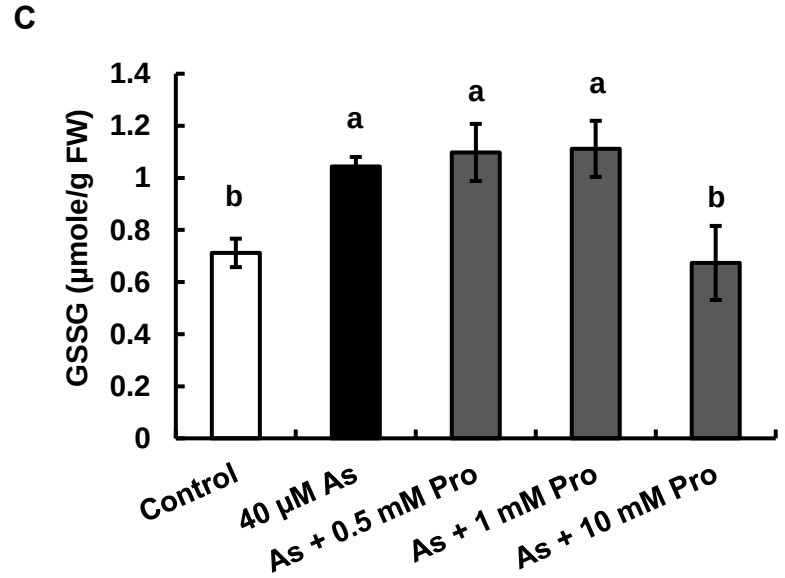

C
GSSG (µmole/g FW)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
a
a
b
b
Control
40 µM As
As + 0.5 mM Pro
As + 1 mM Pro
As + 10 mM Pro

Figure 4.3 Glutathione content at 40 μM arsenate (As)-stressed BY-2 cells in presence of different concentration of exogenous proline (Pro). Total GSH (A), reduced glutathione (GSH) (B), and oxidized glutathione (GSSG) (C) content in As-unadapted tobacco BY-2 cells induced by Pro under AsO_4^- stress. Values represent the mean ±SD (n=3)

4.4.4 *Effects of exogenous proline on the activity of arsenate reductase in arsenate-stressed BY-2 cells*

Fig. 4.4 shows the effect of proline on the activities of arsenate reductase in tobacco BY-2 cells under AsO_4^- stress. In the presence of arsenate, compared with control, 0.5 mM and 1 mM proline did not show any effect on arsenate reductase activity, whereas, 10 mM proline decreased it. At 60 μM AsO_4^-, proline at 10 mM did not show any effect on arsenate reductase activity compared with arsenate stress.

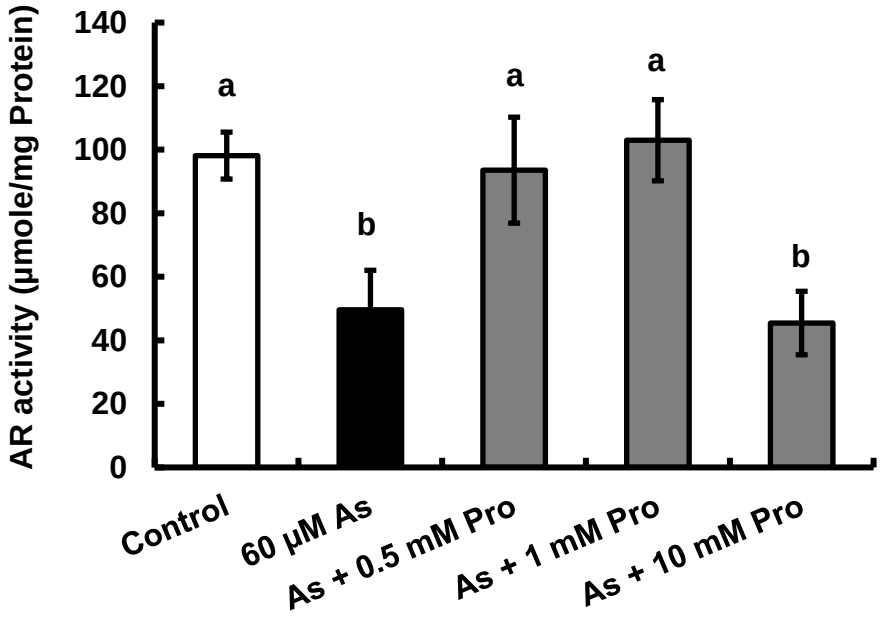


Figure 4.4 Arsenate reductase (AR) activity of arsenate (As) unadapted tobacco BY-2 suspension cells induced by proline (Pro) under AsO_4^- stress. Four-day-cultured cells were co-treated with 0.5 mM, 1 mM and 10 mM exogenous Pro respectively at 60 μM AsO_4^- stress medium. Means ± SD of three independent experiments is shown.

4.4.5 *Superoxide dismutase activity*

Arsenate stress significantly increased the SOD activity in tobacco BY-2 cells represented in Fig. 4.5. The SOD activity in control condition is lower than the AsO_4^- stress. In the presence of AsO_4^-, proline at 0.5 mM to 10 mM did not show any effect on the SOD activity compared with control. On the other hand, exogenous proline at 0.5 mM, to 10 mM decreased the activity of SOD compared with AsO_4^- stress.

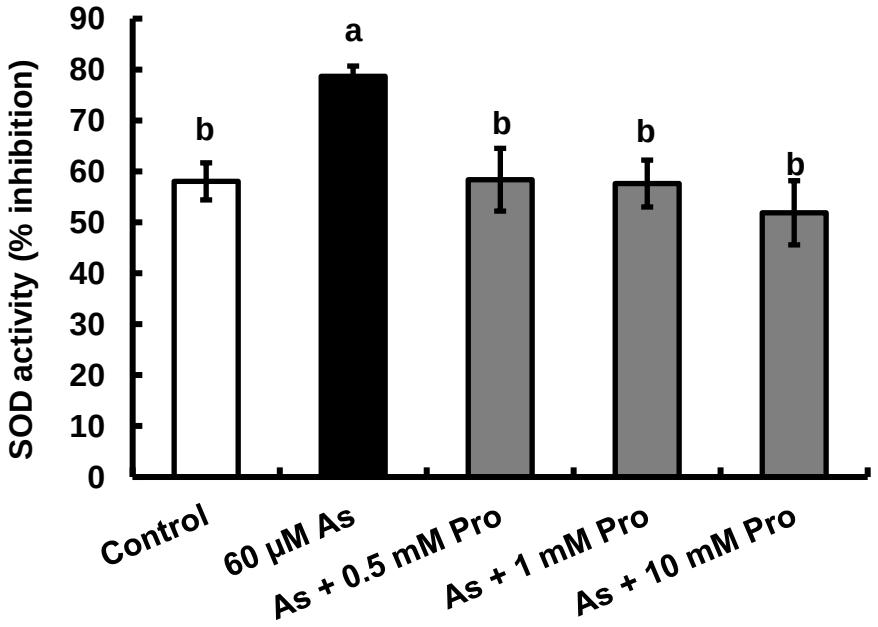


Figure 4.5 Superoxide dismutase (SOD) activity of arsenate (As)-unadapted tobacco BY-2 suspension cells induced by higher concentration of proline (Pro) under AsO_4^- stress. Suspension cells of a 7-day-old culture were inoculated into different fresh media, and cells were collected at 4-days after inoculation. Values represent the mean ±SD (n=3).

4.4.6 Effects of exogenous arginine *on BY-2 cell growth in the absence of arsenate*

To investigate the roles of exogenous arginine for the mitigation of arsenate stress in BY-2 cells, we examined whether exogenous arginine shows any effects on BY-2 cells. We measured the fresh weight (FW) and dry weight (DW) of cells at 0, 4, 6, and 8 days after

inoculation (DAI) in response to exogenous arginine in the absence of arsenate. We found that 0.5 mM, 1 mM and 10 mM arginine did not change BY-2 cell growth compared with control. (Fig. 4.6A and B).

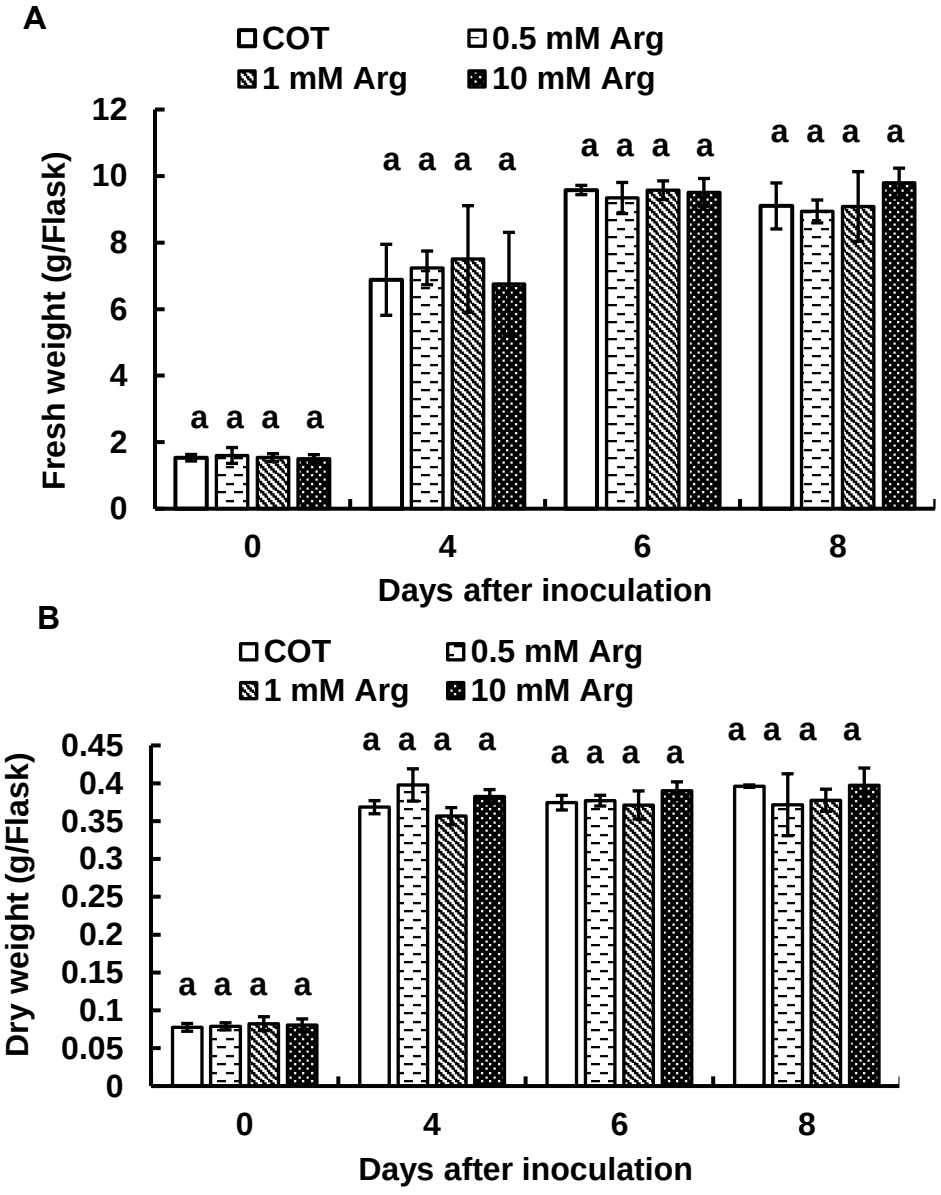
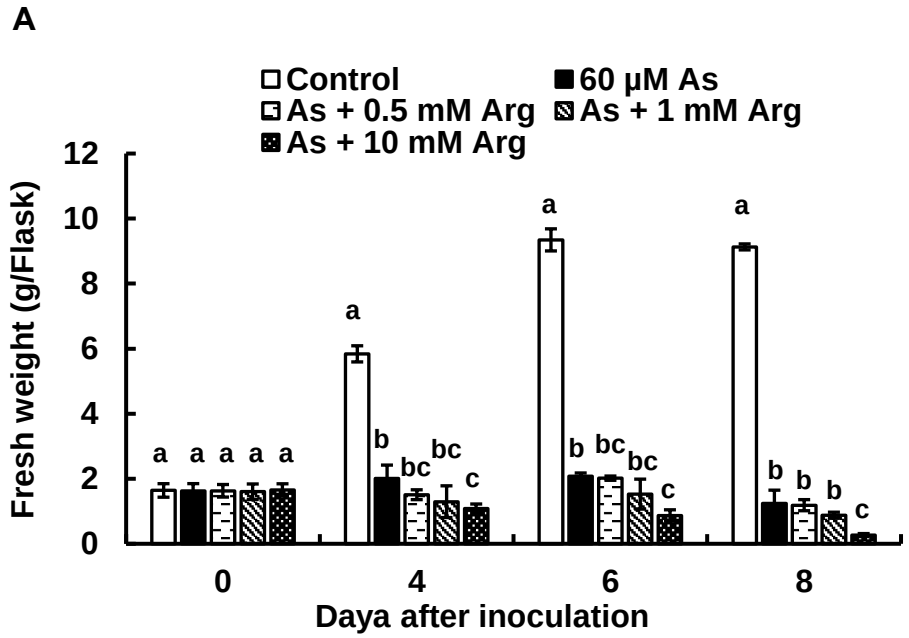


Figure 4.6 Effects of exogenous arginine (Arg) on BY-2 cell growth. A, Shows the cell growth based on fresh weight and B, shows the cell growth based on dry weight in response to 0.5 mM, 1 mM, and 10 mM Arg at 0, 4, 6, and 8 days after inoculation. Averages of cell growth from three independent experiments (n = 3) are shown. Error bars represent SE. Based on *p* values obtained in the *t*-test, there were no significant differences ($p<0.05$) between control

(untreated) cells and treated cells in fresh weight or dry weight at each time point after inoculation.

4.4.7 *Effects of exogenous arginine on the inhibition of BY-2 cell growth by arsenate*

To investigate whether exogenous arginine recovered the inhibition of cell growth by arsenate, we examined the effects of exogenous arginine on the growth of BY-2 cells cultured at 60 μM AsO_4^- stress. The FW and DW of cells at 0, 4, 6, and 8 days after inoculation were measured. In the presence of 60 μM AsO_4^-, 0.5 mM and 1 mM arginine did not affect the cell growth but 10 mM arginine inhibited it (Fig. 4.7A and B). Moreover, AsO_4^- at 60 μM induced more inhibition of cell growth in the presence of exogenous arginine than in the absence of exogenous arginine. These results suggest that exogenous arginine, like proline, did not mitigate the arsenate-induced growth inhibition of BY-2 cells but enhances the sensitivity of BY-2 cells to arsenate.



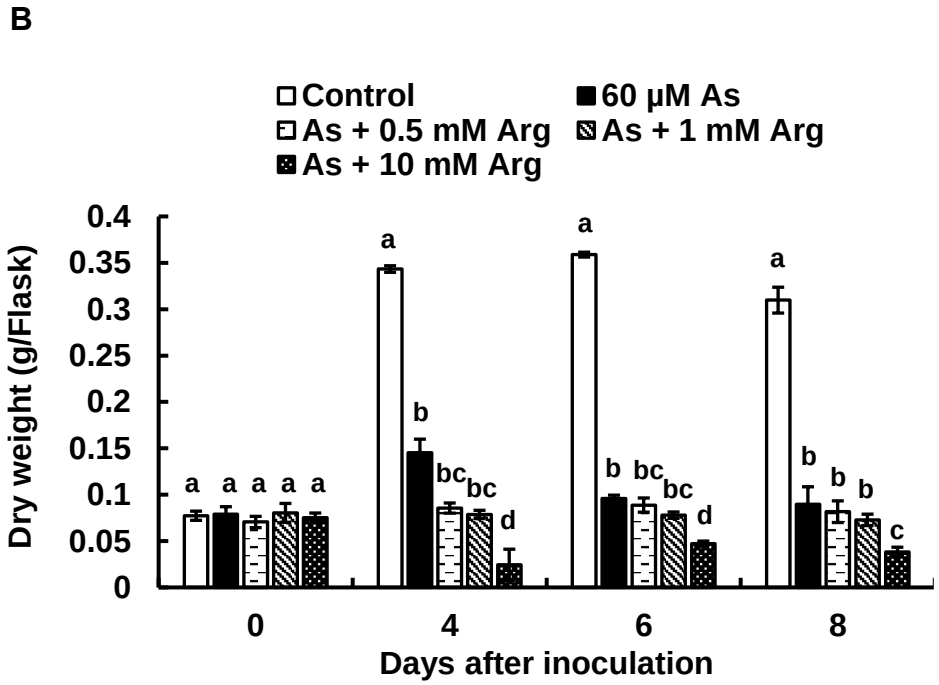


Figure 4.7 Effects of exogenous arginine (Arg) on 60 µM arsenate (As)-stressed BY-2 cells. Reduction of BY-2 cell growth by arsenate (Fresh weight basis, A; dry weight basis, B) in the presence of 10 mM Arg but not in the presence of 0.5 mM or 1 mM Arg at 4, 6 and 8 days after inoculation. Averages of cell growth from three independent experiments (n = 3) are shown. The error bars represent SE. For the same inoculation day, values indicated by the same letter do not differ significantly at 5% level of significance as determined by Tukey's test.

4.4.8 *Effects of exogenous alanine on BY-2 cell growth in the absence of arsenate*

We tested the effect of exogenous alanine in BY-2 cells growth to find out whether exogenous alanine mitigates arsenate induced growth inhibition of BY-2 cells. We measured the FW and DW of cells at 0, 2, 4, 6, and 8 DAI in response to exogenous alanine in the absence of arsenate. We found that 0.5 mM to 10 mM alanine did not show any effect on BY-2 cell growth compared with control. (Fig. 4.8A and B).

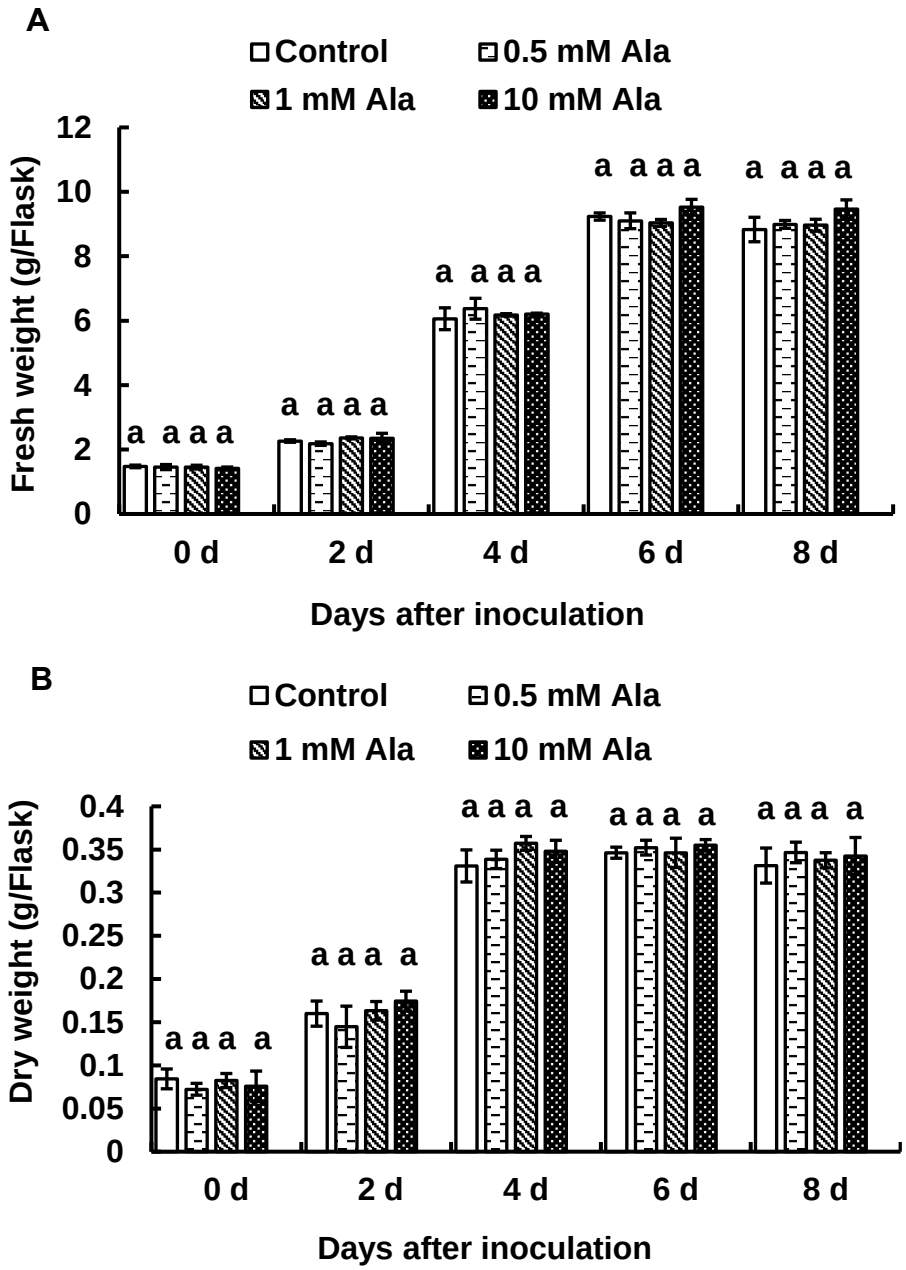


Figure 4.8 Effects of exogenous alanine (Ala) on BY-2 cell growth. A, Shows the cell growth on the basis of fresh weight and B, shows the cell growth on the basis dry weight in response to 0.5 mM, 1 mM, and 10 mM Ala at 0, 2, 4, 6, and 8 days after inoculation. Averages of cell growth from three independent experiments (n = 3) are shown. Error bars represent SE. Based on *p* values obtained in the *t*-test, there were no significant differences ($p<0.05$) between control (untreated) cells and treated cells in fresh weight or dry weight at each time point after inoculation.

4.4.9 *Effects of exogenous alanine on AsO_4^- induced BY-2 cell growth*

We examined the effects of exogenous alanine on AsO_4^- induced BY-2 cell cultured under 60 μM AsO_4^- stress. The FW and DW of cells at 0, 4, 6, and 8 DAI were measured. In the presence of AsO_4^-, 0.5 mM and 1 mM alanine showed a similar effect on BY-2 cell growth compared with 60 μM AsO_4^- stress but 10 mM alanine significantly recovered the AsO_4^- induced growth inhibition (Fig. 4.9A and B). These results suggest that exogenous alanine mitigate the arsenate-induced growth inhibition of BY-2 cells.

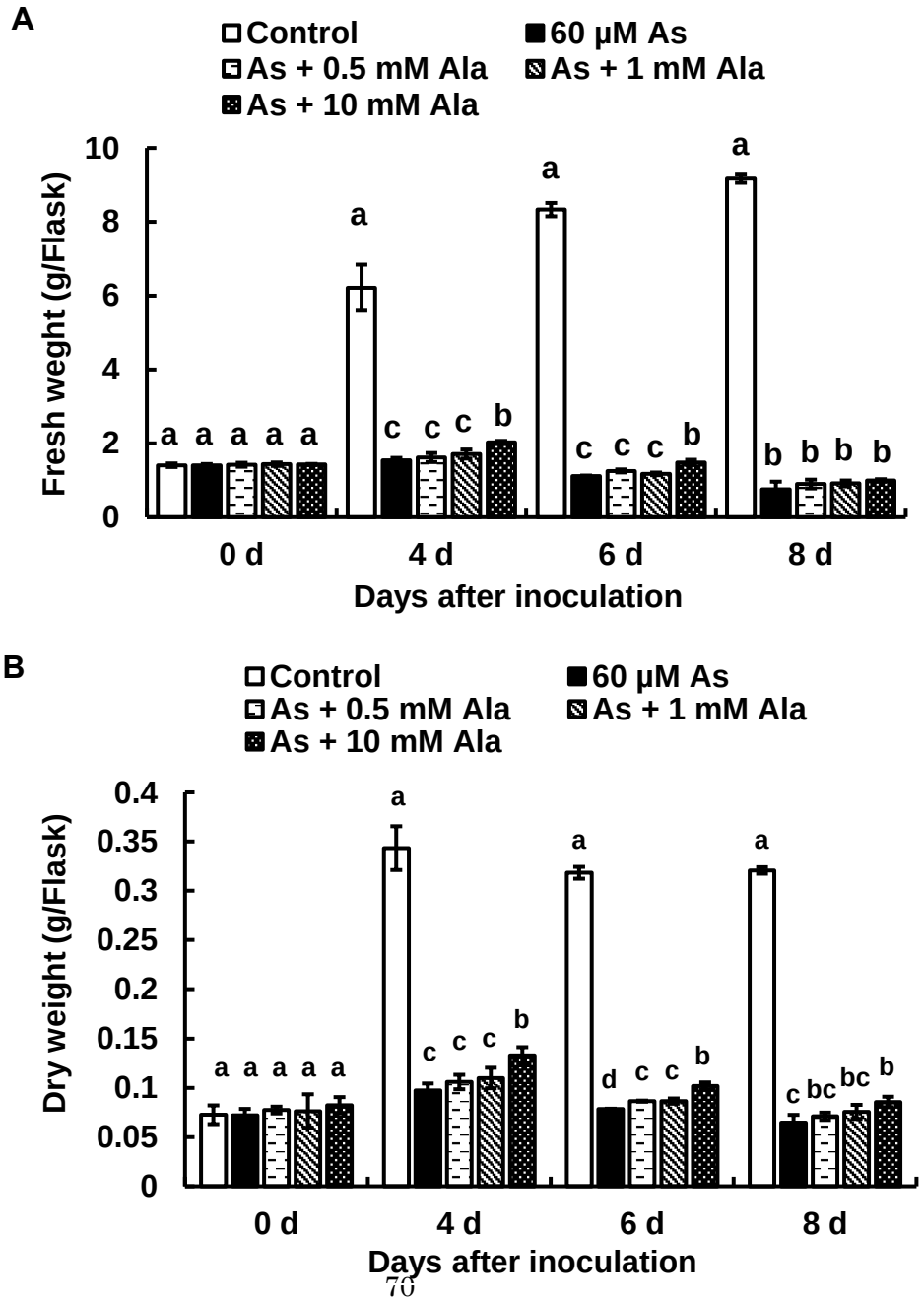
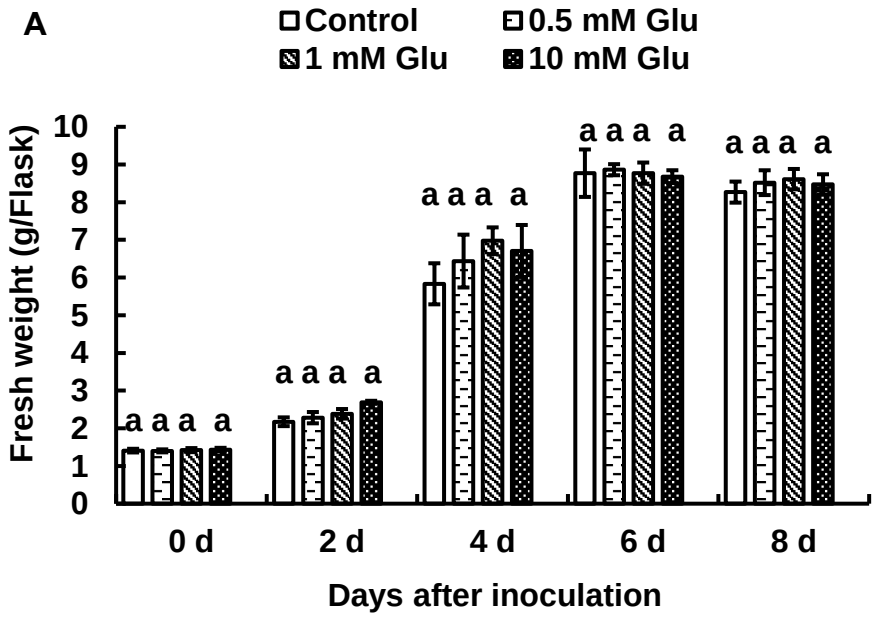


Figure 4.9 Effect of exogenous alanine (Ala) on 60 µM arsenate (As)-stressed BY-2 cells. Increment of BY-2 cell growth by 10 mM Ala (Fresh weight basis, A; dry weight basis, B) but not in the presence of 0.5 mM or 1 mM Ala at 4, 6 and 8 days after inoculation under AsO_4^- stress. Averages of cell growth from three independent experiments (n = 3) are shown. The error bars represent SE. For the same inoculation day, values indicated by the same letter do not differ significantly at 5% level of significance as determined by Tukey's test.

4.4.10 *Effects of exogenous glutamate on BY-2 cell growth*

We examined the effect of exogenous glutamate to see whether glutamate has any effect on BY-2 cell growth (Fig. 4.10). We measured the FW and DW of cells at 0, 2, 4,6, and 8 DAI in response to exogenous glutamate in the absence of arsenate. We found that 0.5 mM to 10 mM glutamate did not show any effect on BY-2 cell growth compared with control (Fig. 4.10).



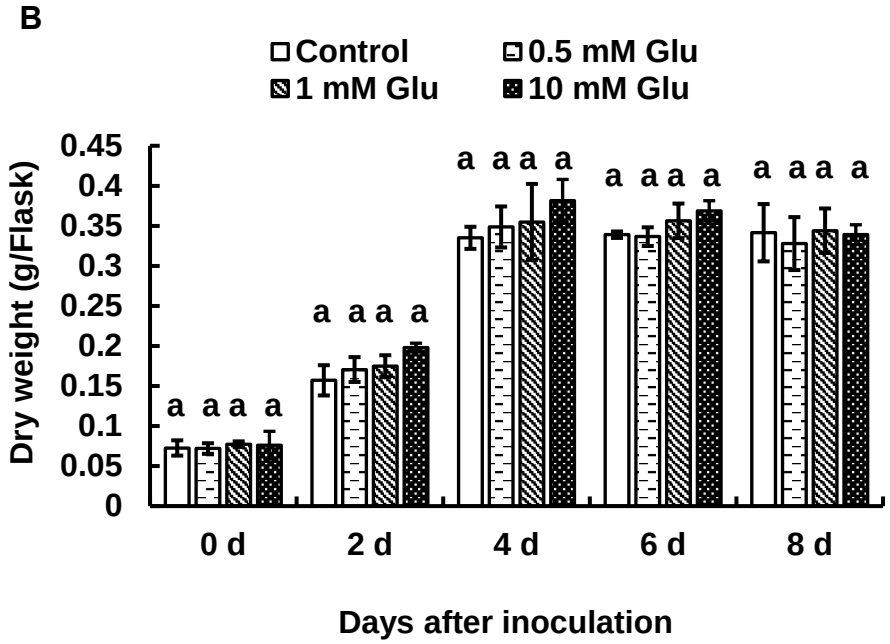


Figure 4.10 Effects of exogenous glutamate (Glu) on BY-2 cells. A, Shows the cell growth on the basis of fresh weight and B, shows the cell growth on the basis dry weight in response to 0.5 mM, 1 mM, and 10 mM Glu at 0, 2, 4, 6, and 8 days after inoculation. Averages of cell growth from three independent experiments (n = 3) are shown. Error bars represent SE. Based on *p* values obtained in the *t*-test, there were no significant differences ($p<0.05$) between control (untreated) cells and treated cells in fresh weight or dry weight at each time point after inoculation.

4.4.11 *Effects of exogenous glutamate on the inhibition of BY-2 cell growth by arsenate*

Figure 4.11 shows that in the presence of arsenate, exogenous glutamate at 0.5 mM, 1 mM and 10 mM did not affect BY-2 cell growth as well as did not improve arsenate-induced cell growth reduction. These results suggest that unlike proline effects, the arsenate in the presence of glutamate did not enhance the cell growth reduction.

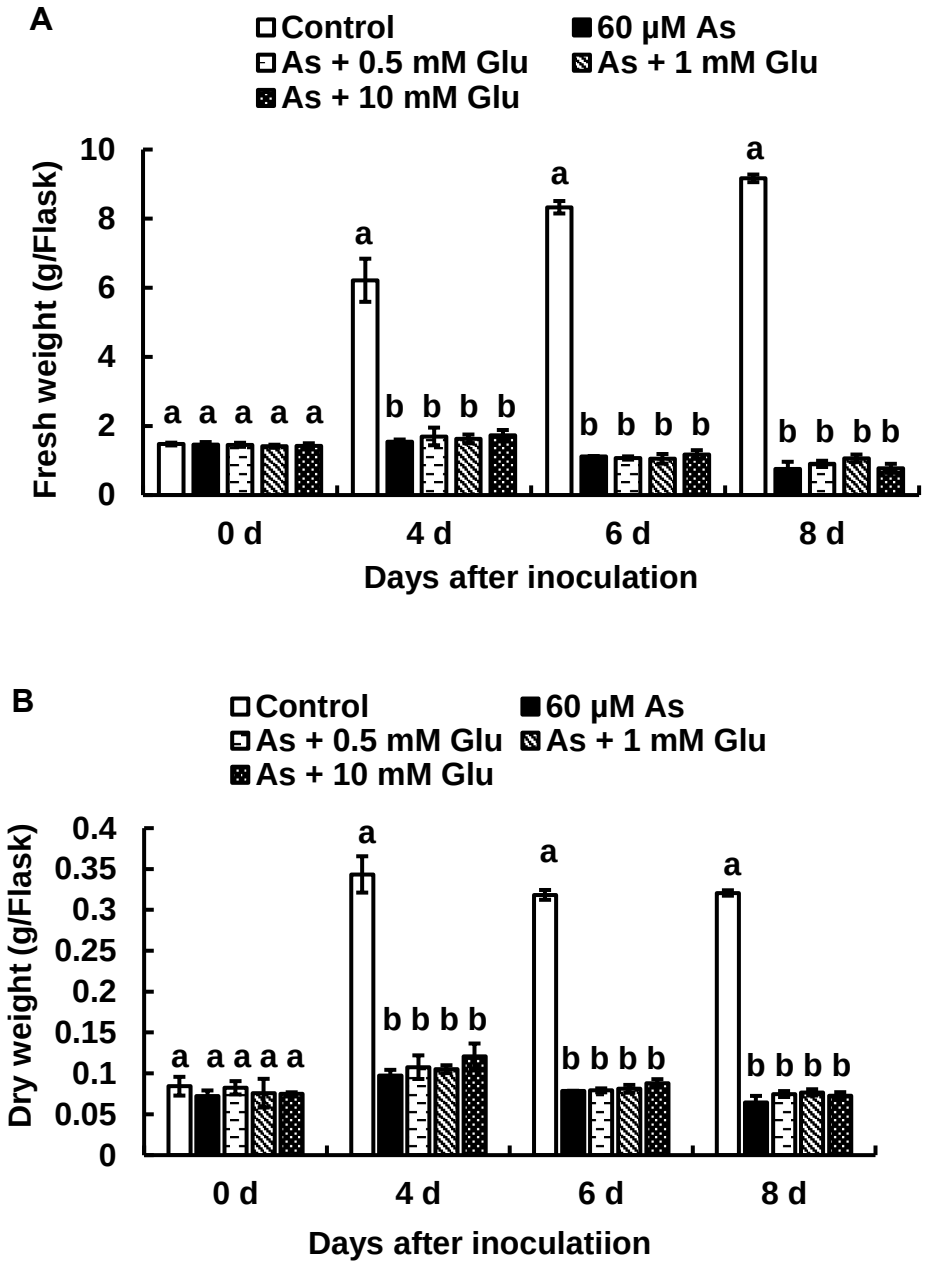


Figure 4.11 Effects of exogenous glutamate (Glu) on 60 μM arsenate (As)-stressed BY-2 cells. In the presence of As, Glu at 0.5 mM, 1 mM and 10 mM (Fresh weight basis, A; dry weight basis, B) did not show any effect on BY-2 cell growth compared with AsO_4^- stress. Averages of cell growth from three independent experiments (n = 3) are shown. The error bars represent SE. For the same inoculation day, values indicated by the same letter do not differ significantly at 5% level of significance as determined by Tukey's test.

4.4.12 *Effects of exogenous betaine on BY-2 cell growth in the absence of arsenate*

To investigate the roles of exogenous betaine for the mitigation of arsenate stress in BY-2 cells, we examined whether exogenous betaine shows any effects on BY-2 cells. We measured the FW and DW of cells at 0, 4, 6, and 8 DAI in response to exogenous betaine in the absence of arsenate. We found that 0.5 mM and 1 mM betaine did not change BY-2 cell growth compared with control but 10 mM betaine drastically decreased the cell growth (Fig. 4.12A and B).

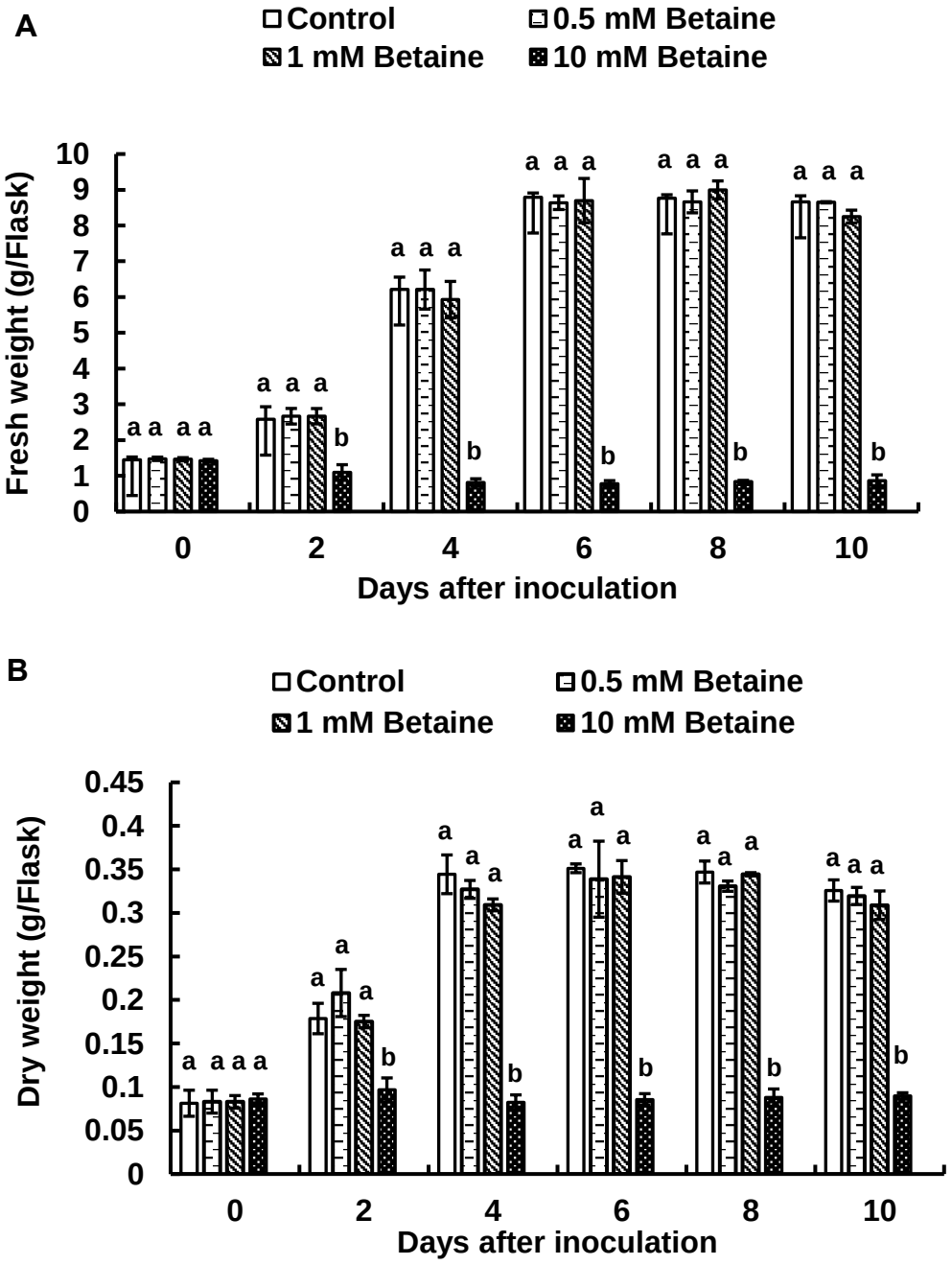
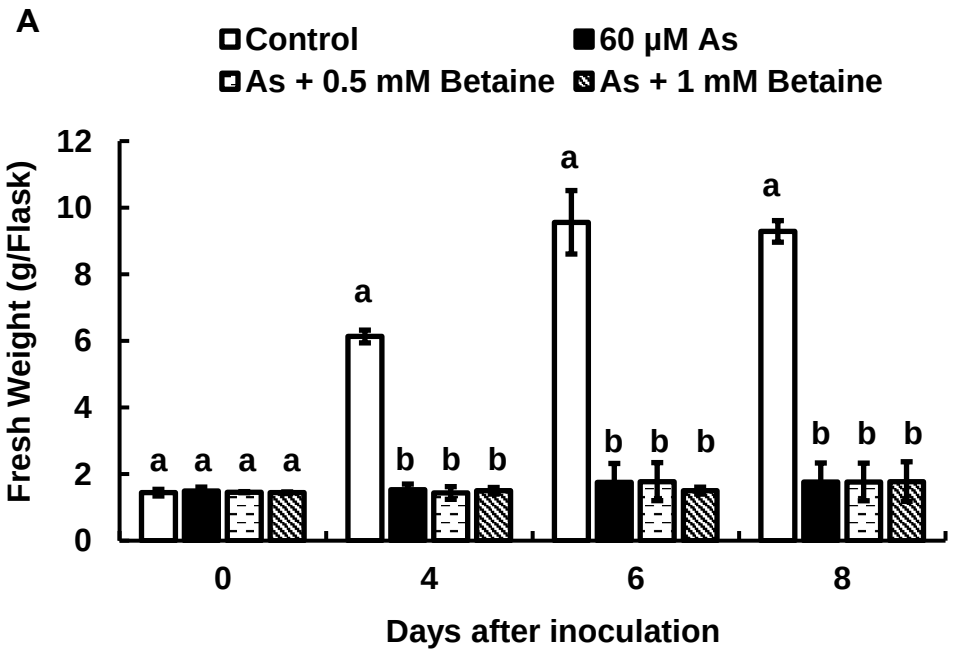


Figure 4.12 Effects of exogenous betaine on BY-2 cell growth. A, Shows the cell growth based on fresh weight and B, shows the cell growth based on dry weight in response to 0.5 mM, 1 mM, 10 mM and 20 mM betaine at 0, 2, 4, 6, 8 and 10 days after inoculation. Averages of cell growth from three independent experiments (n = 3) are shown. Error bars represent SE. Based on *p* values obtained in the *t*-test, there were no significant differences (*p<0.05*) between control (untreated) cells and treated cells in FW or DW at each time point after inoculation.

4.4.13 *Effects of exogenous betaine on the inhibition of BY-2 cell growth by arsenate*

Figure 4.13 shows that exogenous betaine at 0.5 mM and 1 mM did not affect BY-2 cell growth as well as did not improve arsenate-induced cell growth reduction. These results suggest that the arsenate in the presence of betaine did not enhance the cell growth reduction.



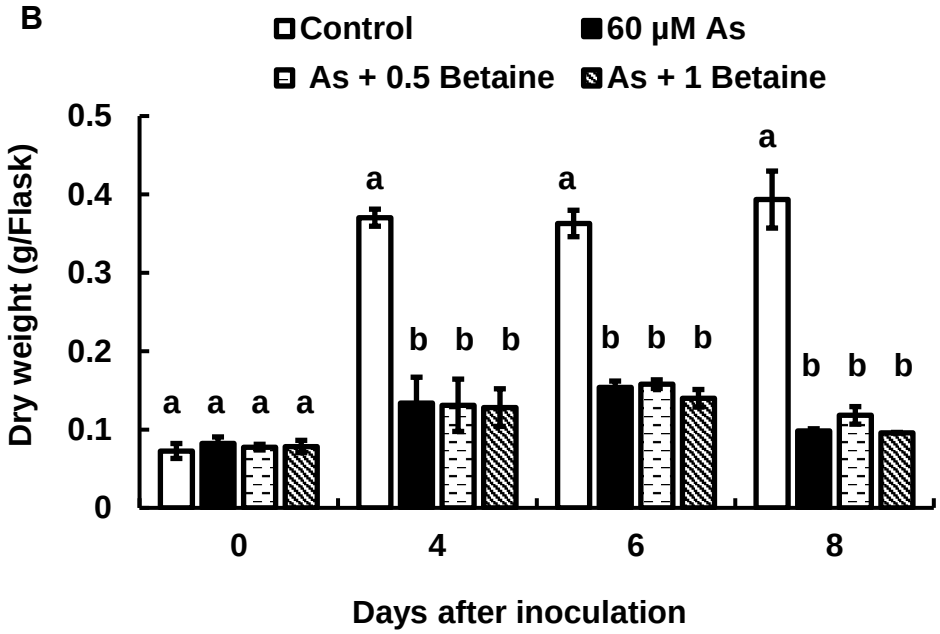


Figure 4.13 Effects of exogenous betaine on 60 µM arsenate (As)-stressed BY-2 cells. Exogenous betaine at 0.5 mM and 1 mM (Fresh weight basis, A; dry weight basis, B) showed similar effect on BY-2 cell growth at AsO_4^- stress. Averages of cell growth from three independent experiments (n = 3) are shown. The error bars represent SE. For the same inoculation day, values indicated by the same letter do not differ significantly at 5% level of significance as determined by Tukey's test.

4.4.14 *Effects of exogenous proline on lipid peroxidation of tobacco BY-2 cells in absence and presence of arsenate*

We measured the MDA, a product of lipid peroxidation, contents in BY-2 cells to investigate the protective effect of exogenous proline against AsO_4^- induced oxidative damage (Fig. 4.14). At 60 µM AsO_4^- stress, BY-2 cells did not show an increased level of MDA (Lipid peroxidation) content compared with control (Fig.4.14A). Exogenous proline at 0.5 mM, 1 mM and 10 mM did not show any effect on MDA content in BY-2 cells in absence of AsO_4^-

(Fig. 4.14A). In the presence of arsenate, 1mM and 10 mM exogenous proline significantly decreased the MDA content in BY-2 cells compared with AsO_4^- stress only.

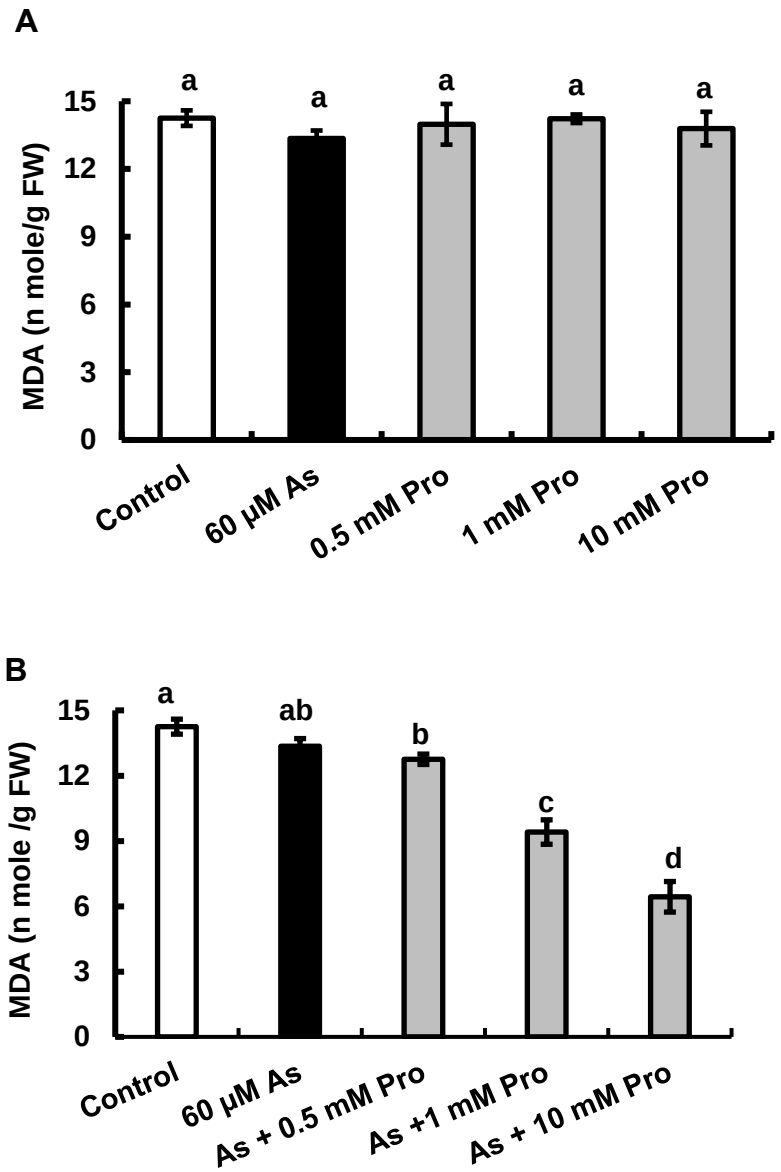


Figure 4.14 Effects of exogenous proline (Pro) on lipid peroxidation at 60 µM AsO_4^--stressed BY-2 cells. Exogenous Pro effect on MDA content in BY-2 cells cultured at 0.5 mM, 1 mM and 10 mM Pro (A, in absence of arsenate, B, in presence of arsenate stress). Averages of three independent experiments (n = 3) are shown. The error bars represent SE. For the same inoculation day, values indicated by the same letter do not differ significantly at 5% level of significance as determined by Tukey's test.

4.5 DISCUSSION

Arsenic, the most toxic metalloid, widely concerned and distributed in the environment. Plants take up arsenic mainly as arsenate. Exposure to arsenate causes considerable stress in plants, including inhibition of growth (Stoeva and Bineva, 2003), physiological disorders (Stoeva et al., 2005) and finally death. It was reported that proline mitigates heavy-metal toxicity by different mechanisms in plants. However, whether proline mitigates AsO_4^- stress in BY-2 cells are to be investigated. In the previous study, we have presented the AsO_4^--induced growth inhibition of BY-2 cells and the increasing rate of growth inhibition by that arsenate in the presence of higher proline. In the present study, we examine that higher proline decreased the glutathione content and did not show the effect on arsenate reductase activity as well as decreased the activity of SOD. We also investigated the effects of some stress-related organic molecules on the BY-2 cell growth both under stress and non-stress conditions to compare the results with proline effects, as well as to clarify the proline-enhanced cell growth reduction by arsenate.

In the present study, the proline content was increased both under arsenic-stressed and non-stressed conditions in a concentration-dependent manner in response to the application of exogenous proline (Fig. 4.1). It was reported that GSH content is significantly increased in plants upon arsenic exposure (Srivastava et. al., 2007). Our data also showed that total GSH content is increased in AsO_4^--stressed BY-2 cells (Fig. 4.2A), which indicates that GSH is an important element that regulates stress-induced changes in plants. In this study, we also found that the total GSH, GSH and GSSG contents are decreased in BY-2 cells under arsenate stress condition in presence of exogenous proline (Fig. 4.2A, B and C). On the other hand, Agarwal et al, (2011) reported that GSH content is increased in proline-treated bean (*Phaseolus vulgaris*) plants that mitigate selenium stress. Previous reports suggest that the increased level of glutathione pool is generally regarded as a protective response against oxidative stress (May and Leaver, 1993; Noctor and Foyer, 1998) and

glutathione-deficient plants are hypersensitive to arsenate (Li et al., 2006). To further clarify with this, we investigated the effect of proline at 40 μM AsO_4^- stress (Fig. 4.3). In the presence of 40 μM AsO_4^-, neither 0.5 mM nor 1 mM proline affected the glutathione content in BY-2 cell but 10 mM proline decreased total GSH, GSH and GSSG compare with AsO_4^- stress. Though it seems contradictory according to our findings but the response of GSH level in terms of mitigation of stress is similar to our findings. It is well established that the detoxification of As and Cd requires GSH and PCs in plants (Howden et al., 1995; Shi et al., 1996; Cobbet et al., 1998; Dhankher et al., 2002; Verbruggen et al., 2009). Agarwal (2011) reported that selenium stress is mitigated by increasing GSH level as well as we found that GSH level is decreased and did not mitigate stress. It is reported that the decreased level of GSH hampers the cell division as well as GSH-deficient plants are sensitive to heavy metal stresses such as copper and cadmium (Xiang et al., 2001). It is also reported that GSH content is enhanced in some metal-tolerant plants, such as *Arabidopsis trichome* (Gutiérrez et al., 2000) and *Sedum alfredii* (Sun et al., 2007).

The depletion of GSH level in plants may, therefore, increase the susceptibility of BY-2 cells. Similar to our findings, Xiang et al. (2001) reported that Arabidopsis plants with low GSH level were hypersensitive to cadmium stress due to the limited capacity of these plants to make PCs, as well as the GSH-deficient mutant *cad2-1* was also found to be more sensitive to AsO_4^- (Li et al., 2006). Therefore, the present investigation suggests that the decrease of total GSH and GSH by exogenous proline application may increase the sensitivity of BY-2 cells to AsO_4^-. Therefore, the present investigation suggests that the decrease of both total GSH and GSH by proline at higher concentration may increase the sensitivity of BY-2 cells to AsO_4^-.

In this study, compared with control, exogenous proline at 10 mM decreased the arsenate reductase activity at AsO_4^- stress condition. Our data showed that exogenous proline at 10 mM did not show any effect on arsenate reductase activity compared with

arsenate stress (Fig. 4.4). It is reported that the reduction of arsenate to arsenite is the main aspect of arsenic detoxification in plants (Zhang et al., 2002) which accelerates the conversion of arsenate to arsenite, leading detoxification and sequestration of arsenite. These results suggest that in the presence of arsenate, exogenous proline did not detoxify arsenate from BY-2 cells.

Previous studies reported that the antioxidant enzymes (e.g. SOD) are important for plants to defend the oxidative stress caused by arsenate (Cao et al., 2004; Srivastava et al., 2005). In this study, arsenate stress significantly increased the SOD activity compared with control. In the presence of arsenate, proline at 10 mM decreased the SOD activity compared with AsO_4^- stress (Fig. 4.5). Previous reports indicated that antioxidant enzyme system would be activated to avoid the oxidative damages effectively. It is also reported that PCs and anti-oxidative enzymes are considered as an important defense system to detoxify heavy metals and metalloids (Sneller et al., 1999; Schmöger et al., 2000). Considering the previous reports, our present study suggests that arsenate stress enhances the damaging effect of BY-2 cell by decreasing the SOD activity in presence of exogenous proline.

In our findings, arsenate decreased the glutathione pool in the presence of proline, and proline did not show increasing activity of arsenate reductase as well as decreased the SOD activity in the presence of arsenate. Although further investigation is necessary to elucidate the enhancement of sensitivity to arsenate by proline, there is a possible reason as follows (Fig. 4.15). Arsenate is converted to arsenite by arsenate reductase and arsenite can act as an inhibitor of pyrroline-5-carboxylate dehydrogenase (P5CDH) (Nirenberg and Jakoby, 1960; Strecker, 1960), which is a member of the superfamily of aldehyde dehydrogenases. On the other hand, proline is catabolized to cytotoxic glutamate-5-semialdehyde (GSA)/pyrroline-5-carboxylate (P5C) by proline dehydrogenase (ProDH) and then GSA/P5C is converted to glutamate by P5CDH (Deuschle, 2001). Taken together, BY-2 cells can produce GSA/P5C due to the catabolism in response to application of proline but cannot

eliminate the catabolites due to the inhibition of P5CDH under arsenate stress condition.

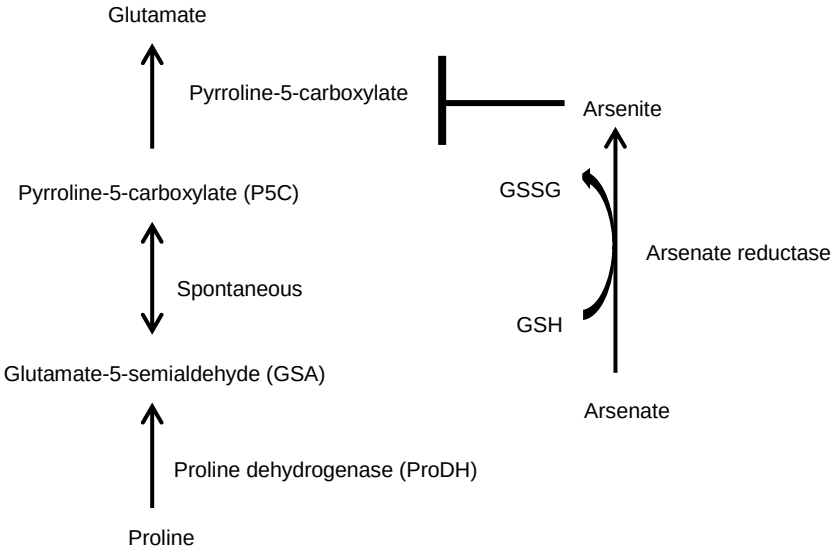


Figure 4.15 A hypothetical flow-diagram showing the inhibition of proline catabolic process by arsenite. Proline is degraded to glutamate catabolizing by the central enzymes ProDH and P5CDH. Arsenite inhibits P5CDH activity and hence accumulates P5C/GSA which is toxic to cells. GSH, reduced glutathione; GSSG, oxidized glutathione.

Plants have evolved various potential mechanisms to protect against the adverse effects of heavy metal toxicity. Amino acids and their derivatives are also functions as a metal chelator that conferred to plants resistance to toxic levels of metal ions (Manara, 2012). It is reported that the concentrations of amino acid in plants under arsenic chronic stress play a vital role. Arsenic toxicity altered the amino acid content and antioxidant activity (Dwivedi et al., 2010). The mechanism of 10 mM proline-enhanced negative effects of arsenate is novel one, which is also important to understand mitigatory roles of proline in BY-2 cells. In this study, we investigated the effects of some stress-related organic molecules on the BY-2 cell growth both under arsenate stress and non-stress conditions to clarify the proline-enhanced cell growth reduction by arsenate.

The report suggests that there is an interaction between arginine and proline metabolism, especially when they are applied exogenously. Arginine is degraded to proline

and then further catabolized following the metabolic pathway similar to proline (Thompson, 1980). In the present study, we found that arginine at 0.5 mM to 10 mM did not affect BY-2 cell growth (Fig. 4.6) but in the presence of AsO_4^-, like 10 mM proline, 10 mM arginine inhibited the growth of BY-2 cell and the inhibition is more pronounced than AsO_4^- stress only (Fig. 4.7). As exogenously applied arginine showed a similar effect, like proline, on BY-2 cell growth suggesting that arginine followed the same catabolic pathway like proline and also enhances the sensitivity of BY-2 cell growth to arsenate.

Arsenic is non-essential and generally toxic to plants. It is reported that a coordinated response of thiolic ligands and stress-responsive amino acids seems to play a role in arsenic tolerance in plants to achieve the effective complexation of arsenic by PCs (Tripathi et al., 2013). Here, we investigated the effect of exogenous alanine on BY-2 cell growth both at arsenic stress and non-stress condition. Alanine at 0.5 mM to 10 mM did not affect BY-2 cell growth (Fig. 4.8). In the present of AsO_4^-, neither 0.5 mM alanine nor 1 mM alanine affected BY-2 cell growth but 10 mM alanine significantly ameliorates the AsO_4^- induced growth inhibition (Fig. 4.9). Previous reports suggest that alanine help in the regulation of intracellular pH without causing phytotoxicity (Sousa and Sodek, 2002), and Thakur and Rai (1985) observed that exogenous application of alanine delayed wilting under stress conditions in maize. Alanine, unlike proline, followed separate catabolic pathway and the previous report indicated that alanine removes the feedback inhibition of proline synthetic pathway, therefore increased the accumulation of proline during stress condition (Zhang et al., 1995; Hong et al., 2000). It is well known that during stress condition proline accumulation is necessary for the mitigation of stresses. As alanine, does not follow catabolic pathway similar to proline, arsenate did not show any inhibitory actions. Together, these results indicated that exogenous alanine mitigates arsenate induced growth inhibition.

It is well known that glutamate is a common biosynthetic precursor of proline (Moat et al., 2003). In the present study, exogenous glutamate at 0.5 mM, 1mM and 10 mM did not

show any effect on BY-2 cell growth compared with control (Fig.4.10). In the presence of arsenate, glutamate at 0.5 mM to 10 mM did not improve arsenate induced growth inhibition of BY-2 cell (Fig. 4.11). These results suggest that glutamate did not mitigate the AsO_4^- induced BY-2 cell growth reduction.

Betaine has been accumulated by plants and studied extensively as a compatible solute. Exogenous application of betaine increased the tolerance of plants to abiotic stress (Chen and Murata, 2008). However, the protective effect of betaine against arsenate toxicity in BY-2 cells needs to be investigated. Exogenous betaine at 0.5 mM and 1 mM did not show any effect on BY-2 cell growth but significantly inhibited by 10 mM (Fig. 4.12). At AsO_4^- stress, both 0.5 mM and 1mM betaine did not improve arsenate induced growth inhibition of BY-2 cell (Fig. 4.13). Though exogenous betaine has been shown to mitigate NaCl- or Cd -induced growth inhibition of tobacco BY-2 cells (Hoque et al., 2007; Islam et al., 2009), our results suggests that betaine did not mitigate the AsO_4^- induced cell growth reduction.

In conclusion, exogenous proline plays dynamic roles in BY-2 cells under arsenic stress, which depends on its level of concentration. In our study, exogenous proline at higher concentration did not mitigate arsenate stress and further sensitizes the BY-2 cells to arsenate. Though our previous reports have been shown that exogenous proline suppress cell death and confer tolerance to NaCl stress and Cd stress (Haque et al., 2007, 2008; Banu et al., 2009; Islam et al., 2009) but exogenous proline at higher concentration did not ameliorate arsenate stressed BY-2 cells growth inhibition. The enhancement of stressing effect by arsenic stress in the presence of higher proline will open new insights for further studies of stress mitigation in plants.

SUMMARY

The toxic metalloid arsenic is widely distributed in soil environment and causes physiological and structural disorders in plants. The reduction of crop yield by arsenic stress has been recognized as a threat to the sustainable food production. Arsenic occurs predominantly as inorganic forms such as arsenate (AsO_4^-) and arsenite (AsO_3^-). Plants take up arsenic mainly as arsenate. Proline is accumulated as a compatible solute in plants under various stress conditions. Exogenous proline scavenges free radicles, improves plant metabolism and stimulates plant growth under stress conditions. However, in some cases, exogenous proline showed its toxicity to plants and caused programmed cell death. It is reported that proline ameliorates heavy-metal toxicity in plants. However, the role of proline in arsenate-stressed BY-2 cells remains unclear. In this study, I investigated the effects of exogenous proline on tobacco BY-2 cells cultured under AsO_4^- stress and found that proline depending on its concentrations plays dynamic roles in BY-2 cells such as mitigation of arsenate stress in response to lower concentrations of proline (e.g., 0.05 mM), whereas sensitization of BY-2 cells to arsenate in response to higher proline treatment (e.g., 10 mM).

The effects of exogenous proline, exogenous arsenate, and the co-treatment of arsenate and proline on BY-2 cells growth, cell death, and cell number. Here, AsO_4^- significantly inhibited the growth of BY-2 cells at 60 μM but not at either 40 μM or 50 μM. Proline at 0.05 mM to 10 mM did not affect the cell growth but delayed it at 20 mM. Therefore, for mitigating the arsenic stress, I examined the effects of a wide range (0.05, 0.1, 0.5, 1, and 10 mM) of exogenous proline on the inhibition of cell growth by 60 μM arsenate and found that proline at 0.05 mM alleviated the arsenate-induced cell growth inhibition, but surprisingly accelerated the growth inhibition at 1 mM and 10 mM. Proline at 0.05 mM and 0.1 mM significantly decreased the number of Evans Blue stained cells but 10 mM proline boosted the number of stained cells. Proline at 0.05 mM and 0.1 mM increased the total

number of cells, whereas 10 mM proline decreased the total number of cells. These results indicate that the effects of 0.05 mM proline on arsenate-stressed BY-2 cells reversed with the increase of proline concentration to 10 mM, and also suggests that the lower concentration of proline mitigates arsenate stress, whereas the higher concentration of proline sensitizes BY-2 cells to arsenate. To insight into the issue that 10-mM proline enhances the sensitivity of BY-2 cells to arsenate, I further investigated the effects of 10 mM proline on BY-2 cells treated with 40 μM arsenate and found that 40 μM arsenate did not inhibit cell growth in the absence of proline but inhibits it in the presence of proline, suggesting that application of 10 mM proline enhances the adverse effects of arsenate. Together, these results suggest that proline plays two distinct roles in BY-2 cells in the presence of arsenate.

The molecular mechanisms of the lower proline in BY-2 cells under arsenate stress were described. The mechanisms of how proline mitigates stress in plants are not fully understood but appear to involve its chemical properties and effects on redox systems such as the glutathione pool. Glutathione is the major source of non-protein thiols in plant cells and functions as a key component of the antioxidant network. The increased level of glutathione pool is generally regarded as a protective response against oxidative stress. The reduced glutathione content is associated with the protection to oxidative stress in plants. In the presence of AsO_4^-, 10 mM proline decreased the total glutathione content compared with both control and arsenate but not 0.05 mM proline. Furthermore, 0.05 mM proline decreased the reduced glutathione and increased the oxidized glutathione contents compared with arsenate stress, whereas 10 mM proline decreased both reduced and oxidized glutathione levels. These results suggest that during mitigation process proline maintains glutathione homeostasis, whereas, during sensitization of BY-2 cells to arsenate, the glutathione pool is decreased.

Arsenate is readily reduced to arsenite through arsenate reductase using reduced

glutathione as a reductant. Here, at AsO_4^- stress condition, compared with control, 0.05 mM proline did not show any effects on arsenate reductase activity but 10 mM proline decreased it. Compared with arsenate stress, 0.05 mM proline significantly increased the arsenate reductase activity, but 10 mM proline did not. These results suggest that proline mitigates arsenate stress by increasing the arsenate reductase activity which accelerates the conversion of arsenate to arsenite, leading detoxification and sequestration of arsenite. The activity of antioxidant enzymes is important for stress mitigation. It is also reported that the SOD activity is decreased at AsO_4^- stress condition in *Glycine max* (Chandrakar et al., 2016). It is reported that the SOD activity is increased at AsO_4^- stress condition. Here, proline at 0.05 mM and 0.1 mM did not show any effect on the SOD activity in the presence of arsenate. On the reverse, exogenous proline at 10 mM decreased the activity of SOD compared with AsO_4^- stress only.

The above data indicate that 10 mM proline inversely regulates all the parameters those were positively regulated by 0.05 mM proline, and enhances stressing effects. The mechanism of 0.05 mM proline-induced stress mitigation is consistent with the previous reports. However, the mechanism of 10 mM proline-enhanced negative effects of arsenate is novel one, which is also important to understand mitigatory roles of proline in BY-2 cells. Although further investigation is necessary to elucidate the enhancement of toxicity of arsenate by proline, there is a possible reason for the enhancement as follows. Arsenate is converted to arsenite by arsenate reductase and arsenite can act as an inhibitor of pyrroline-5-carboxylate dehydrogenase (P5CDH), which is a member of the superfamily of aldehyde dehydrogenases. On the other hand, proline is catabolized to cytotoxic glutamate-5-semialdehyde (GSA)/pyrroline-5-carboxylate (P5C) by proline dehydrogenase (ProDH) and then GSA/P5C is converted to glutamate by P5CDH. Together, BY-2 cells can produce GSA/P5C due to the catabolism in response to application of proline but cannot eliminate the catabolites due to the inhibition of P5CDH under arsenate stress condition.

I investigated the effects of some stress-related organic molecules on the BY-2 cell growth both under arsenate-stress and non-stress conditions to compare the results with proline effects, as well as to clarify the proline-enhanced cell growth reduction by arsenate. It has been reported that arginine and proline are synthesized independently but not degraded independently. There is a considerable interconnection between their catabolic pathways when applied exogenously. Arginine is degraded to proline and then further catabolized following the metabolic pathway similar to proline. Here, I found that 10 mM arginine did not affect BY-2 cell growth, but in the presence of arsenate, like 10 mM proline, 10 mM arginine enhanced the cell growth reduction by arsenate. As arginine follows proline degradation pathway, so the effects of arsenate in BY-2 cells in the presence of proline or arginine also same. This result also strengthens the arsenite-induced P5CDH inhibitory mechanism of negative effects by arsenate. Alanine, unlike proline, follows separate catabolic pathway and alanine inhibits arginine degradation process. Here, exogenous alanine did not affect BY-2 cell growth at 0.5 mM to 10 mM. In the presence of AsO_4^-, neither 0.5 mM nor 1 mM alanine affected the cell growth but 10 mM alanine significantly recovered the AsO_4^--induced growth inhibition. These results suggest that exogenous alanine mitigates arsenate stress. As alanine, does not follow catabolic pathway similar to proline, arsenate did not show any inhibitory actions. Betaine is a compatible solute that accumulates in plants and mitigates stress. Here, I found that exogenous betaine at 0.5 mM and 1 mM did not affect BY-2 cell growth and did not improve arsenate-induced cell growth reduction. Unlike proline effects, the arsenate in the presence of betaine did not enhance the cell growth reduction, suggesting that arsenate, particularly in the presence of proline, increased the sensitivity of BY-2 cells.

CONCLUSIONS

Proline plays dual roles in BY-2 cells under arsenate stress, which depends on its level of concentration. The lower concentration of proline mitigates arsenate stress in BY-2 cells but the higher concentration of proline sensitizes that cells to arsenate.

Proline at 0.05 mM mitigated AsO_4^--induced growth inhibition of BY-2 cells by decreasing reduced GSH and increasing oxidized GSH. Proline at 0.05 mM increased the arsenate reductase activity that utilized reduced GSH as a reductant, which leads to the conversion of arsenate to arsenite and detoxification processes. Thus, proline mitigates arsenate stress in BY-2 cells.

Proline at 10 mM decreased the total GSH, reduced GSH and oxidized GSH as well as did not increase the arsenate reductase activity which is responsible for the conversion of arsenate to arsenite. Moreover, 10 mM proline decreased the antioxidant enzyme such SOD activity.

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