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A Regulatory Approach Caused by Cold Acclimation and Arsenic on the Impairment of Root Growth of Rice (*Oryza sativa*)

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Abstract

Development of root growth is impaired by environmental and chemical effectors although the mechanism is not known. Here, we used pot experiment for cultivation of rice (*Oryza sativa*) to identify the factors involved in impaired growth of root and to explore the effects of cold acclimation and arsenic on the amylase and urease activity in root. The amylase activity was increased whenever exposed to cold for 48h and 72h while mild effect occurred after 24h duration. Acclimation to cold in the presence of different concentrations of Na_2HAsO_4 also increased the amylase activity effectively than cold exposure alone; however, higher concentration of Na_2HAsO_4 is potentially involved in this respect. Conversely, root urease activity was impaired and reduced by cold for the above mentioned times. The reduced urease activity was also caused by the combined effect of cold and arsenic compound. Increased amylase activities in response to cold and arsenic might be involved in the survival process. The two effectors are also involved in the impairment of reduced uptake of urea because of the reduced urease activities. The results clearly demonstrate that both effectors produced an adverse environment where the growth and development of roots are impaired.

Keywords: Cold acclimation, arsenic, root growth, adaptive response.

1. Introduction

Rice (*Oryza sativa*) is one of the most important cereal crops in the world. It is a principal food in developing and developed countries. Only 5% of the total production of rice is used in processed foods, industrial products and alcoholic beverages, while 95.0% of world rice production is consumed as an unprocessed food (Rahman *et al*., 2007). Therefore, the development of this plant is an important aspect in plant metabolism as well as in the metabolism of the other heterotrophic organisms. Root development is impaired either by environmental or chemical factors. Temperature fluctuation is a common phenomenon of the atmosphere and is involved in changes of various metabolic functions (Janska *et al*., 2010). It has been revealed that roots and other protected parts are less cold hardy than the aerial parts of the plant (Havis, 1976; Pellet, 1971). Therefore, it is presumably assumed that cold acclimation may have the role in the development of plant roots (Räisänen *et al*., 2009). Evaluation of the cold hardiness of roots during winter is difficult due to the frozen soil and the lack of reliable methods for assessing freezing damage (Chen *et al*., 1983).

Development of plant growth is one of the biological processes mediated by the coordination of the metabolic processes catalyzed by different enzymes (Renaut *et al*., 2006). For example, urease is involved in the degradation of urea to CO_2 and NH_4^+, the higher the degradation of this urea (N-fertilizer), the higher the formation of NH_4^+ thereby utilization of nitrogen in plant (Haque *et al*., 2010; Mérigout *et al*., 2008). Similarly, amylase is involved in the degradation of amylose to monosaccharide. Amylose is an essentially linear molecule composed of α (1- 4)-linked glycosidic chains (Nishi *et al*., 2001). Therefore, the utilization of these products and their involvement is an important aspect in plant metabolism. Efficiency of nitrogen use is evaluated in terms of development of efficient photosynthetic machinery involving biosynthesis of proteins which mediates the various metabolic steps in the chloroplast (Younis *et al*., 2008). The other aspects of the nitrogen use efficiency are the overall leaf growth, canopy development, light interception and contribution to total photosynthesis (Yildirim *et al*., 2007). All these metabolic activities eventually determine the biomass of plant. The biological processes regarding the development of the plant are directly or indirectly modulated by natural heavy or light elements. Arsenic (As) has been identified to be toxic to the living organisms (Li *et al*., 2007). Prolonged exposure of arsenic has detrimental effects in tissues. It may impair the glycolysis as well as the oxidative processes (Tchounwou *et al*., 2003) and causes different types of pathogenic syndromes in rodents and other organisms. More than 40 million people worldwide are at risk from drinking As-contaminated groundwater (Nordstrom, 2002), and chronic inorganic As poisoning has reached a massive scale in Bangladesh and West

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Bengal, India (Bhattacharjee, 2007). In these countries, As contaminated ground water is also widely used for irrigating crops during dry season for rice production in Bangladesh resulting in arsenic accumulation in soils and elevated arsenic uptake by crops (Meharg and Rahman, 2003; Alam and Sattar, 2000). Elevated arsenic accumulation in rice has the potential to become a new disaster for the population in Southeast Asia (Meharg, 2004). Arsenic concentration in rice grain is often high enough to cause concern even in uncontaminated soils containing background levels of As, because paddy rice appears to be particularly efficient in As assimilation compared with other cereal crops (Williams *et al*., 2007). It is therefore crucial that the mechanism of arsenic accumulation in rice is understood to counteract this widespread contamination of the food chain. The roots of plants are involved in the acquisition of water and nutrients, anchorage of the plant, synthesis of plant hormones and storage functions. Therefore, to fully understand the morphogenesis of roots, it is necessary to analyze the activity of amylase and urease to clarify their role in paddy development and how these enzymes are regulated by environmental adverse effectors like cold and toxic arsenic.

2. Materials and Methods

2.1. Soil collection and pot preparation

The soil was collected from the rice field of Rajshahi University Campus, Bangladesh and kept in several plastic pots. The unwanted materials like stones, gravels, pebbles, plant roots, etc. were removed from the bulk soil. For this experiment, four plastic pots were used; the size of each pot was 70 cm in diameter and 24 cm in height. An adequate amount of soil was taken in each plastic pot. Then sufficient amount of water was poured into each pot and kept for overnight and mixed well. Then the pots were ready for seedling of germinated rice.

2.2. Seed germination

For the germination of seeds (*Oryza sativa*), the following points were carried out: (i) the strongest seeds were selected; the seeds were added to water and floating seeds were discarded; (ii) the seeds were kept in water with temperature below 37^0C overnight; (iii) the seeds were swollen by water absorption and were expected to be effective for germination; (iv) the seeds were seeded in the pots prepared with soil and the efficiency of seed germination was about 90%.

2.3. Cold acclimation and arsenic treatment

After 10 days of germination, the four different pots were described as control, cold, arsenic (1 mM) plus cold and arsenic (10 mM) plus cold. Control pot was used for 24h, 48h and 72h treatments in the room temperature without cold acclimation. The second pot was used for 24h, 48h and 72h duration in the cold chamber and given cold exposure (4~8^0C) with full aeration. In the third pot, paddies were treated with arsenic (Na_2HAsO_4. $7H_2O$, BDH Chemical Ltd.) (1 mM) and kept similarly in cold for 24h, 48h and 72h in the cold chamber. The fourth pot containing paddy was similarly treated with arsenic (10 mM) and kept in cold for 24h, 48h and 72h in the cold chamber. After 10 days of germination, paddies were ruptured consecutively from each pot and the different parts of paddy including root were sampled carefully.

2.4. Assay of amylase and urease activity

One g of paddy root was placed into a mortar with pestle which was kept on ice. It was homogenized with 12 ml of distilled water and centrifuged with 8000 rpm for 10 minutes. The supernatant was collected and used as crude extract. The amylase and urease activities in the crude extract were assayed by the method described by Jayaraman (1981). For amylase and urease activity, 1.0 and 0.1 ml of the crude extract were used respectively. The enzyme activities in root extracts were expressed as µmole/min/mg of protein.

2.5. Assay of protein content in root extract

Roots (1 g) were homogenized with pre-cooled water and were centrifuged at 8000 rpm for 10 min. The supernatants from each root homogenate were used as crude extract for assay of protein by using 100 µl extract. The protein content in root was determined by the procedure of Lowry *et al*. (1951). Briefly, alkaline solution was prepared by mixing 50 ml of alkaline Na_2CO_3 solution (2% Na_2CO_3 in 0.1N NaOH) and 1.0 ml of copper-sodium potassium tartarate solution (1 g sodium potassium tartarate and 0.5 g $CuSO_4$. $5H_2O$ were dissolved in 100 ml distilled water). Hundred µl of tissue extract was taken to the test tube and made up to 1 ml with distilled water. For blank, 1 ml water was used in place of tissue extract. Five ml alkaline solution was added to each tube and mixed well. The tubes were allowed to stand for 10 min at room temperature and 0.5 ml of diluted FCR (Commercial FCR was diluted with equal volume of water) was added and mixed well. After 30 min, the absorbance was taken at 650 nm against the blank. The protein content in each root extract was calculated from the standard graph of bovine albumin (1 mg/ml) and is expressed as g/100 g of root weight.

2.6. Statistical analysis

Results of the experiments were expressed as mean and standard error of different groups. The differences between the mean values were evaluated by ANOVA followed by paired *t*-test using SPSS software.

3. Results and Discussion

3.1. Effect of cold and Na_2HAsO_4 on amylase activity

To examine the role of cold exposure and arsenic treatment on the regulation of amylase activity in root of paddy, the plants in the pot were exposed to cold for 24h in the cold chamber. As shown in figure 1**,** the amylase activity for control was 0.0787 ± 0.0099 µmole while for cold treatment; the value was 0.0706 ± 0.0021 µmole$min^{-1}mg^{-1}$ of protein. The activity in root extract was reduced by 10.29% in response to cold only. Whenever, the paddy was exposed to different concentrations of arsenic and cold, the different amylase activities were observed. In this case, the paddy exposed to 1 mM Na_2HAsO_4 and cold had the enzyme activity 0.1538 ± 0.0123 µmole$min^{-1}mg^{-1}$ of protein after 24 hours of treatment. The results indicated that the activity in paddy root in response to

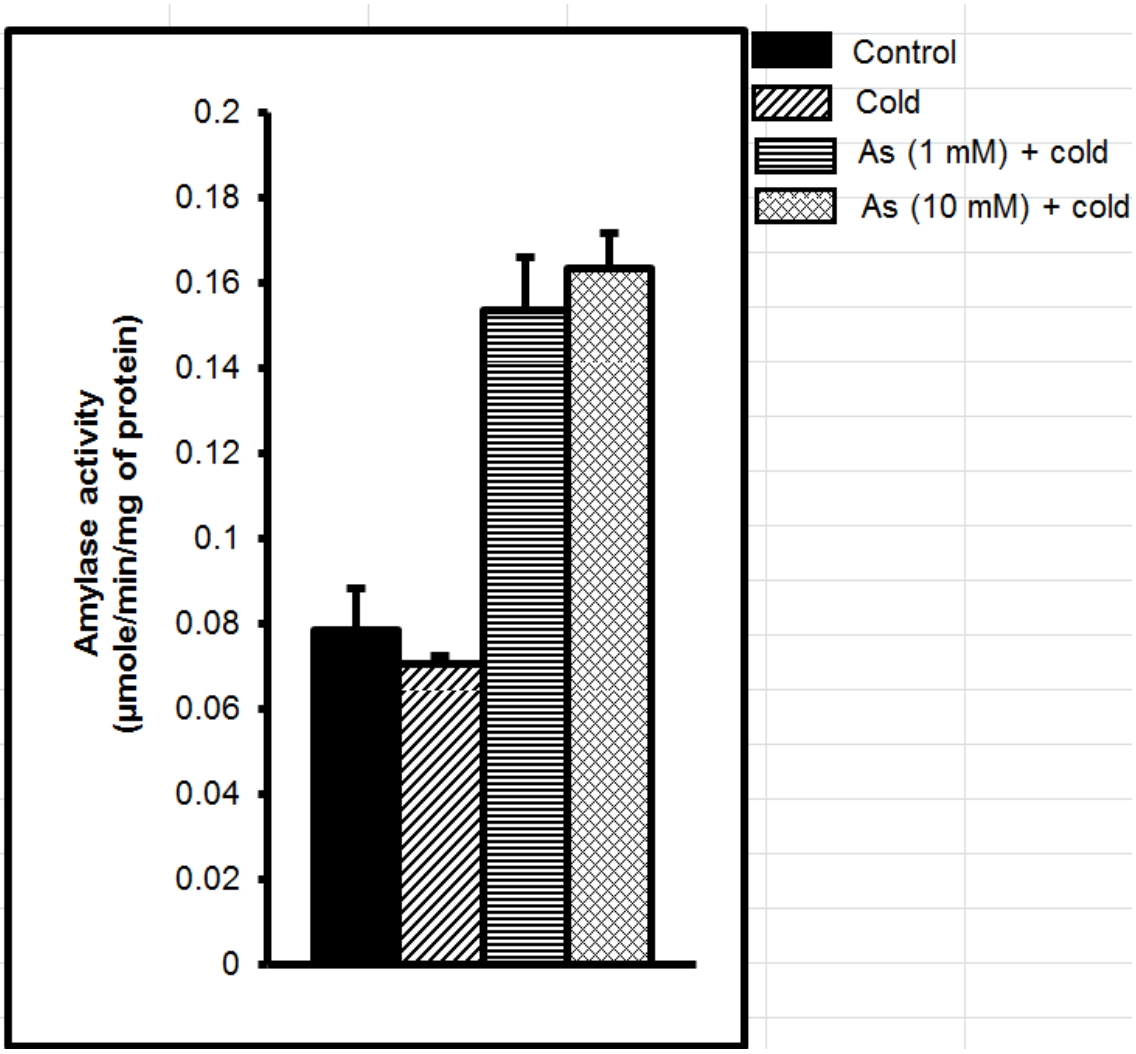

Figure 1. Effect of cold and Na_2HAsO_4 on amylase activity in roots after 24h of treatment. The paddy was treated with different concentrations of arsenic (1 and 10 mM) and kept for 24h in the cold. The paddies in another pot were exposed to cold for 24h only in the cold chamber. Control paddy was similarly used without cold exposure and arsenic treatment. The data are means ± SE for 3 individual measurements in each group.

arsenic had been found to be influenced by 95.42% when compared to control. In addition, the enzyme activity was enhanced by 117.84% when compared to cold exposed paddy. Therefore, it is presumably assumed that short term cold exposure has little impairment on root growth and development; conversely, cold acclimation in presence of arsenic induces a severe effect and stimulates amylase activity in root. In response to 10 mM Na_2HAsO_4 and cold, the amylase activity was found to be 0.1635 ± 0.0085 µmolemin^{-1}mg^{-1} of protein. The results indicated that the activity was increased by 107.75% when compared to control and 131.58% compared to cold exposure only, however, higher concentration of arsenic produces the increased efficiency on enzyme activity. It is evident, both cold acclimation and arsenic are severely involved in inducing the adverse environment where the paddy survives for their growth.

To find the optimum effect of cold acclimation on amylase activity in paddy roots, the extended time was 48h (Fig.2). The cold exposed paddy had amylase activity of 0.0890 ± 0.0116 µmole while for control paddy; the activity was 0.0415 ± 0.0029 µmolemin^{-1}mg^{-1} of protein. The results showed that the activity in root had been increased by 114.45% when they were exposed to cold for 48h when compared to control. The paddy exposed to cold and arsenic (1 mM Na_2HAsO_4) for 48h had enzyme activity of 0.0485 ± 0.0028 µmolemin^{-1}mg^{-1} which indicated that arsenic treated paddy had showed increased activity (16.86%) when compared to control. Moreover, the enhanced enzyme activity (45.50%) was observed in arsenic and cold exposed paddy when compared to cold exposed paddy only. Paddy exposed to cold and arsenic (10 mM Na_2HAsO_4) had urease activity of 0.0380 ± 0.0083 µmolemin^{-1}mg^{-1} of protein. The activity in response to higher dose of arsenic and cold was reduced by 8.43% and 57.30% when compared to control and cold exposed paddy, respectively. The results in figure 2 seem to indicate that the enzyme activities were affected by both cold acclimation and arsenic; however, cold acclimation was critically involved to increase the activity.

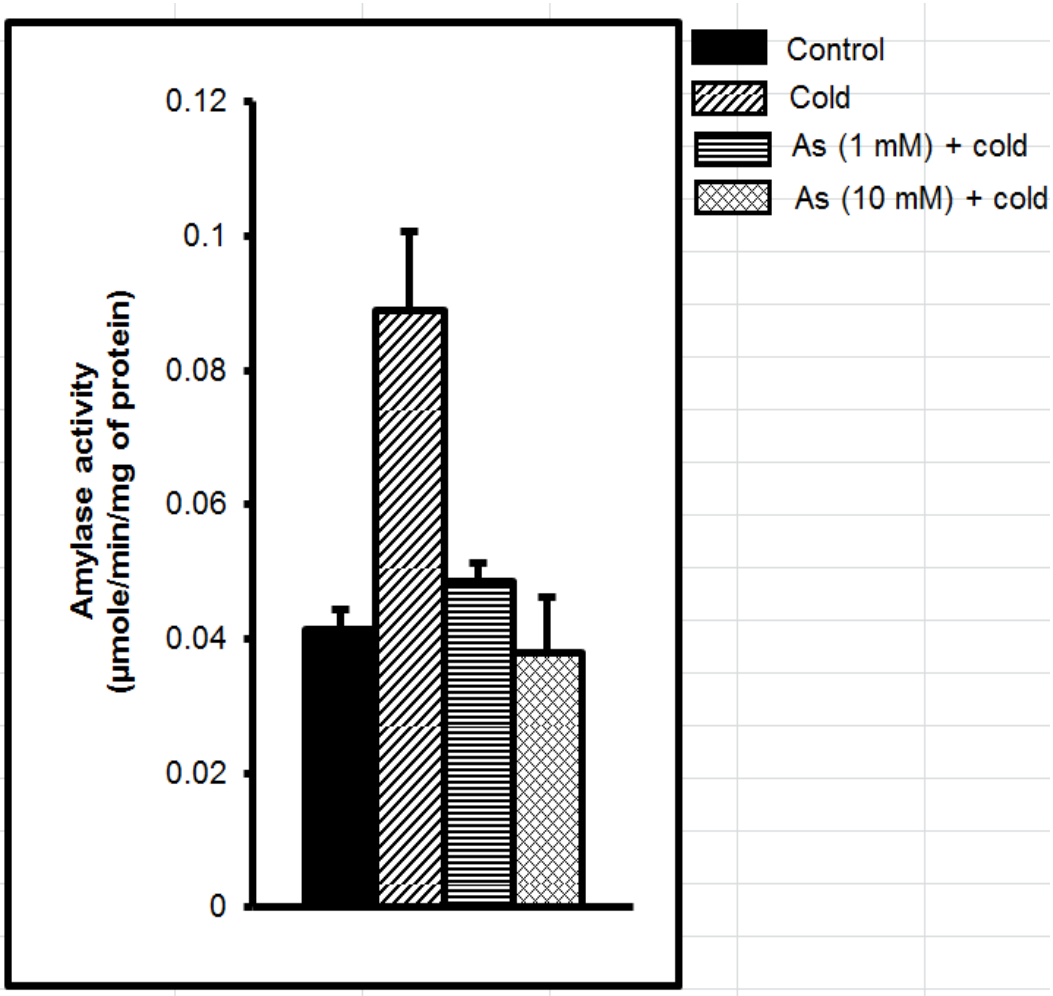

Figure 2. Effect of cold and Na_2HAsO_4 on amylase activity in roots after 48h of treatment. The paddy was treated with different concentrations of arsenic (1 and 10 mM) and kept for 48h in the cold. The paddies in another pot were exposed to cold for 48h only in the cold chamber. Control paddy was similarly used without cold exposure and arsenic treatment. The data are means ± SE for 3 individual measurements in each group.

After 72 hours of treatment, the amylase activities of different types of treated paddy were estimated as 0.0621 ± 0.0046 µmole for control and 0.0698 ± 0.0065 µmole for cold treatment (Fig.3). On the other hand, whenever they were exposed to cold and arsenic (1 mM), the root activity was 0.0757 ± 0.0005 µmolemin^{-1}mg^{-1} .Similar increased effects on root enzyme activities (12.39%) were obtained whenever the plants were exposed to cold while in presence of arsenic, cold acclimation causes similar increased effects on activity by 21.90% when compared to control. However, compared to cold acclimated paddy, arsenic in presence of cold was found to be involved to increase the root enzyme activity by 8.45%. The amylase activity in response to 10 mM Na_2HAsO_4 and cold was found to be 0.0630 ± 0.0059 µmolemin^{-1}mg^{-1} of protein which shows that the enzyme activity was similarly increased by 1.44% when compared to control while reduced by 9.74% as compared to cold exposed paddy. The results indicate that prolonged cold acclimation might be involved in reducing the amylase activity in response to arsenic since growth and development of paddy are retarded after 72h of treatment.

3.2. Effects of cold and Na_2HAsO_4 on urease activity

As shown in figure 4, the urease activities in roots of treated paddy were recorded to determine the effect of cold and arsenic on root growth. After 24 hours of treatment, the root urease activities were estimated as 0.5430 ± 0.0137 µmole for the control and for cold treated paddy, the value was 0.1940 ± 0.0076 µmolemin^{-1}mg^{-1} of protein. Cold acclimation caused a significant decrease in urease activity by 64.27% when compared to the control. However, when paddies were exposed to cold and arsenic (1 mM Na_2HAsO_4) for 24h, the different urease activity was observed and found to be 0.3080 ± 0.0258 µmolemin^{-1}mg^{-1} of protein. The results demonstrate that the urease activity of paddy had been similarly decreased by 43.27% when compared to the control, however, in comparison to cold acclimated paddy, the increased effect (58.76%) on root activity was observed in response to arsenic and cold. The paddy exposed to 10 mM arsenic and cold had urease activity of 0.1990 ± 0.0107 µmolemin^{-1}mg^{-1} showing that the enzyme activity was reduced by 63.35% and increased by 2.57% when compared to control and cold exposed paddy, respectively.

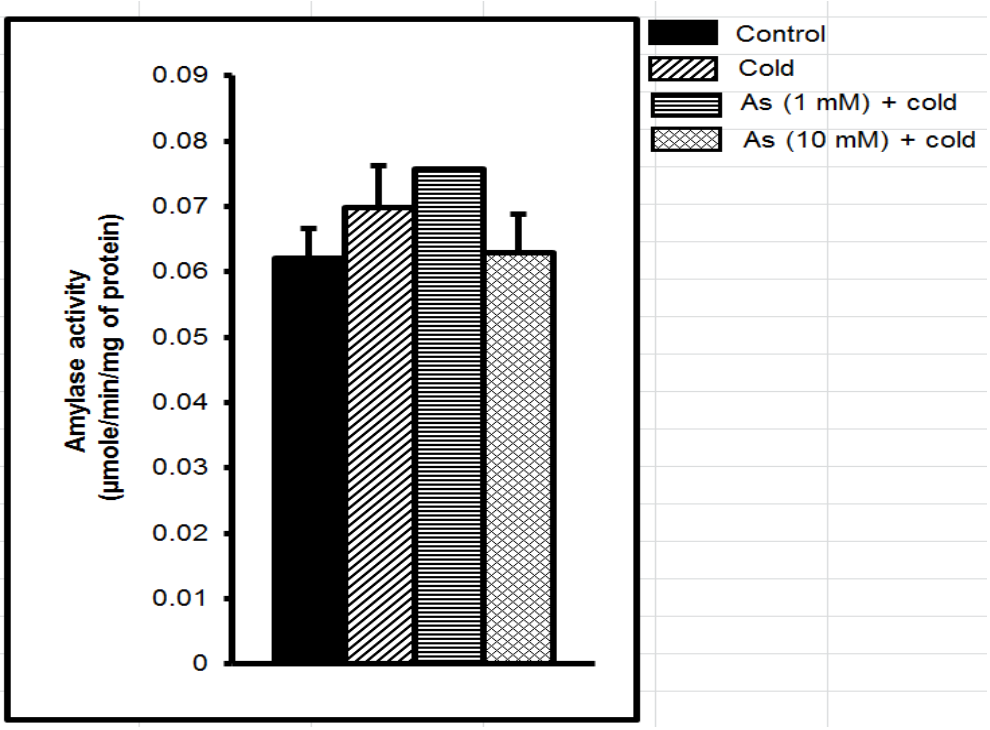

Figure 3. Effect of cold and Na_2HAsO_4 on amylase activity in roots after 72h of treatment. The paddy was treated with different concentrations of arsenic (1 and 10 mM) and kept for 72h in the cold. The paddies in another pot were exposed to cold for 72h only in the cold chamber. Control paddy was similarly used without cold exposure and arsenic treatment. The data are means ± SE for 3 individual measurements in each group.

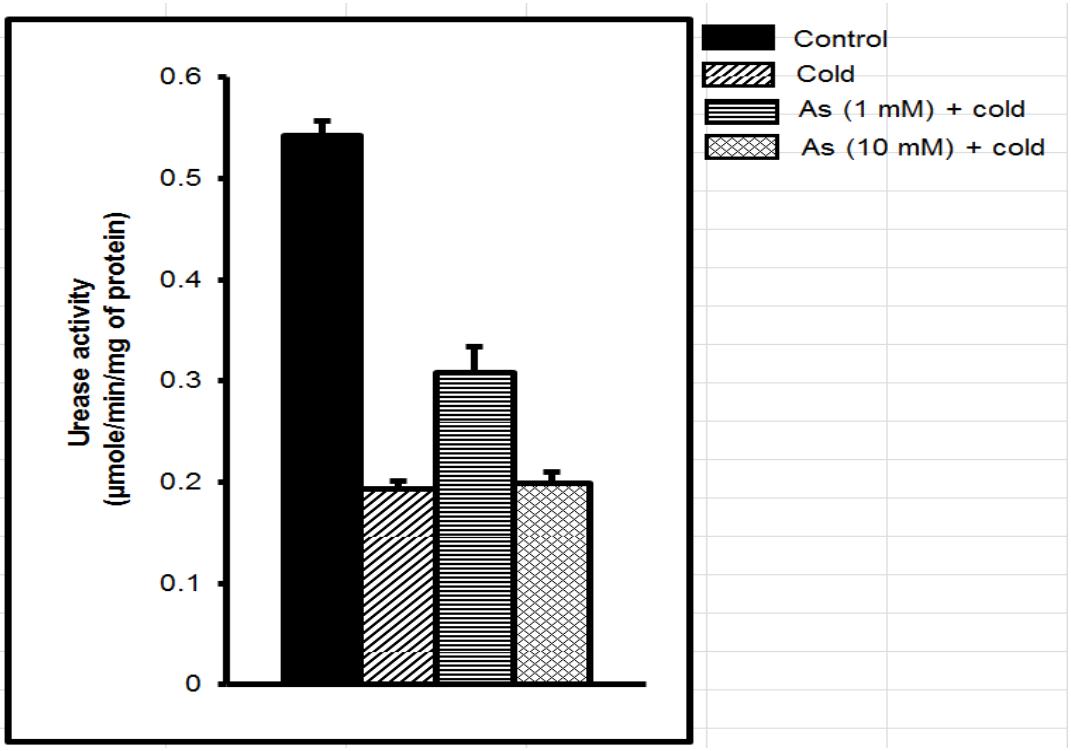

Figure 4. Effect of cold and Na_2HAsO_4 on urease activity in roots after 24h of treatment. The paddy was treated with different concentrations of arsenic (1 and 10 mM) and kept for 24h in the cold. The paddies in another pot were exposed to cold for 24h only in the cold chamber. Control paddy was similarly used without cold exposure and arsenic treatment. The data are means ± SE for 3 individual measurements in each group.

The urease activity might be regulated by the variation of temperature and be strictly followed by the availability of urea in the soil.

To examine the effect of cold on urea induced urease activity, the plants were exposed to cold for 48h along with the combined effect of cold and arsenic. As shown in figure 5, the root urease activities of treated paddy were found to be 0.4950 ± 0.0360 µmole for the control and 0.1180 ± 0.0037 µmole for cold treatment. It was found that the urease activity of paddy root had been reduced by 76.16% for cold acclimation. The paddies exposed to cold and arsenic (1 mM Na_2HAsO_4) had urease activity of 0.1330 ± 0.0077 $\mu molemin^{-1}mg^{-1}$ of protein showing the reduced effect on enzyme activity (73.13%) when compared to control. However, compared to cold exposed paddy, the urease activity was increased by 12.71%. In response to arsenic 10 mM and cold, root urease activity was 0.0670 ± 0.0040 $\mu molemin^{-1}mg^{-1}$ of protein. Addition of higher dose of arsenic also causes the lower effect (86.46%) when compared to control; however, it was lower than the cold exposure alone. Therefore, cold acclimation was directly involved to reduce the urease activity and the effect might be changed in response to the availability of arsenic in the soil. The reduced effect (43.22%) on root activity was occurred by 10 mM arsenic and cold compared to cold acclimated paddy. Therefore, the impairment on urease activity caused by cold acclimation is associated with the administration of arsenic.

Figure 6 shows the effect of cold and different concentrations of arsenic on urease activity of paddy after 72 hours of treatment. Paddies treated with cold had root urease activity 0.2130 ± 0.0348 µmole, whereas 0.1350 ± 0.0127 $\mu molemin^{-1}mg^{-1}$ of protein were recorded for 1 mM Na_2HAsO_4 and cold treatment. The root urease activity of paddy for the control treatment was 0.7240 ± 0.0232 $\mu molemin^{-1}mg^{-1}$ of protein. These results indicate that the root urease activity of paddy had been reduced by 70.58% for cold treatment and 81.35% for arsenic and cold treatment as compared to control. However, compared to cold exposed paddy, the activity was also reduced by 36.61%. Cold acclimation similarly causes the reduced activity in prolonged time and they survive in such critical environment either by lower uptake of soil urea or by other phenomenon. Addition of higher dose of arsenic (10 mM), also seems to be involved in reducing the enzyme activity. Here, the enzyme activity was 0.0400 ± 0.0013 $\mu molemin^{-1}mg^{-1}$ of protein. The results also show that the urease activity was reduced by 81.22% when the paddies were exposed to cold and arsenic compared to cold acclimation. Moreover, 94.47% reduced activity occurred in response to arsenic and cold as compared to the control. Therefore, it is reasonable that both cold and arsenic creates an adverse environment and may reduce the activity because of the lower uptake of urea in the soil.

In our study, we found that the amylase and urease activity in root of paddy had been affected by arsenic and cold treatment. Although 24h treatment with cold did not increase the amylase activity, however, prolonged exposure affected and enhanced its activity and the reduced activity was considered to be the mild effect of cold. In different studies, it was assumed that cold acclimation induces glucose synthesis in plants (Wanner and Junttila, 1999); therefore, enhanced activity of amylase is correlated to their results. It has been revealed that sugars appear essentially in plant during cold acclimation as shown for example by the inability of an Arabidopsis sucrose synthase mutant to cold acclimation (Welling and Palva, 2006) or the requirement for light in low- non freezing temperature-induced cold acclimation connected to sugar accumulation (Wanner and Junttila, 1999, Yadegari *et al*., 2007). Precise function of sugars is not known, but their high abundance in cold acclimated plants suggest a role in osmoregulation and less abundant sugars might also have a role in cryoprotection or as signaling molecules (Welling and Palva, 2006). There is increasing evidence that chilling causes elevated levels of active oxygen species (AOS), which contribute significantly to chilling damage (Wise and Naylor, 1987). AOS such as superoxide (O^{-}_2), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^{-}) and singlet oxygen ($^1O_2$), are present in plants in various at 25 ^oC as a result of normal aerobic metabolism. Therefore, accumulation of sugars during cold acclimation might be correlated to the above mechanisms. Heavy metal accumulation in soil and its importance on the morphological, biochemical and cytological aspects of plants have been considered to be the major issue for the development of plant growth by many workers (Abbasi *et al*., 1992; Prakash *et al*., 2004). The results appeared to be affected more severely whenever the pants were exposed to higher arsenic concentration (10 mM). A number of studies demonstrated the reduced growth of plants (including paddy) grown in soil containing high arsenic or when irrigated with water containing high concentration of arsenic (Smith *et al*., 2001). The data presented in the study show that Na_2HAsO_4 in different doses stimulated the enzyme activity possibly by impairing root growth and development. Stoeva *et al*. (2003) also reported that arsenic accumulated mainly in the root system and to a lesser extent in the overgrown organs, inhibits the growth and fresh and dry biomass accumulation. There is, however, contrasting reports showing that the effect of Na_2HAsO_4 on shoot and root growth is similar (Simon *et al*., 1978). It seems likely that the effect of Na_2HAsO_4 on stem and root growth varies depending on the plant species, level of contamination and plant tissue ability to tolerate Na_2HAsO_4. It is therefore, both cold acclimation and arsenic can be regarded as to be the adverse effectors for the plant growth.

The reduced urease activity in root extract in response to cold was reported in our study. The adverse environment created by cold acclimation for prolonged period was made, therefore, nutritional deficiencies and other cellular defects might be happened in paddy. Promotion of growth is impaired during cold acclimation. Therefore, protein synthesis because of the utilization of nitrogenous substances is impaired and as a result, urease activity should be declined. In addition, deficiency of urea in soil is also associated to this lower activity of urease. In soils, urea is rapidly degraded to ammonium and CO_2 by urease, a nickel-dependent enzyme, which amongst others is synthesized and secreted by microorganisms (Watson *et al*., 1994).

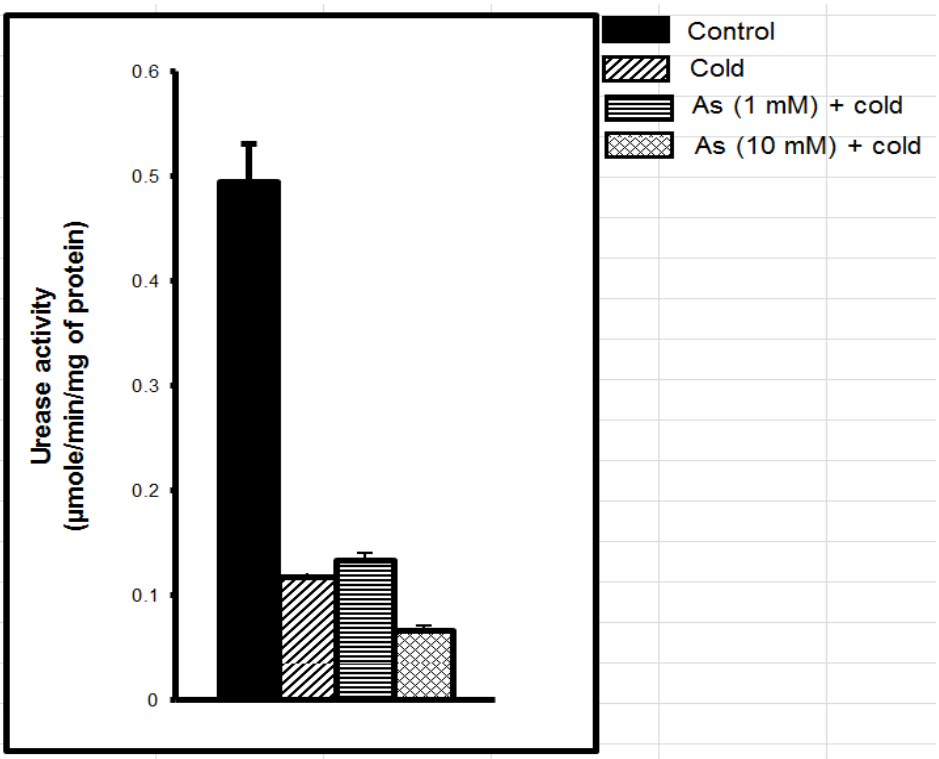

Figure 5. Effect of cold and Na_2HAsO_4 on urease activity in roots after 48h of treatment. The paddy was treated with different concentrations of arsenic (1 and 10 mM) and kept for 48h in the cold. The paddies in another pot were exposed to cold for 48h only in the cold chamber. Control paddy was similarly used without cold exposure and arsenic treatment. The data are means ± SE for 3 individual measurements in each group.

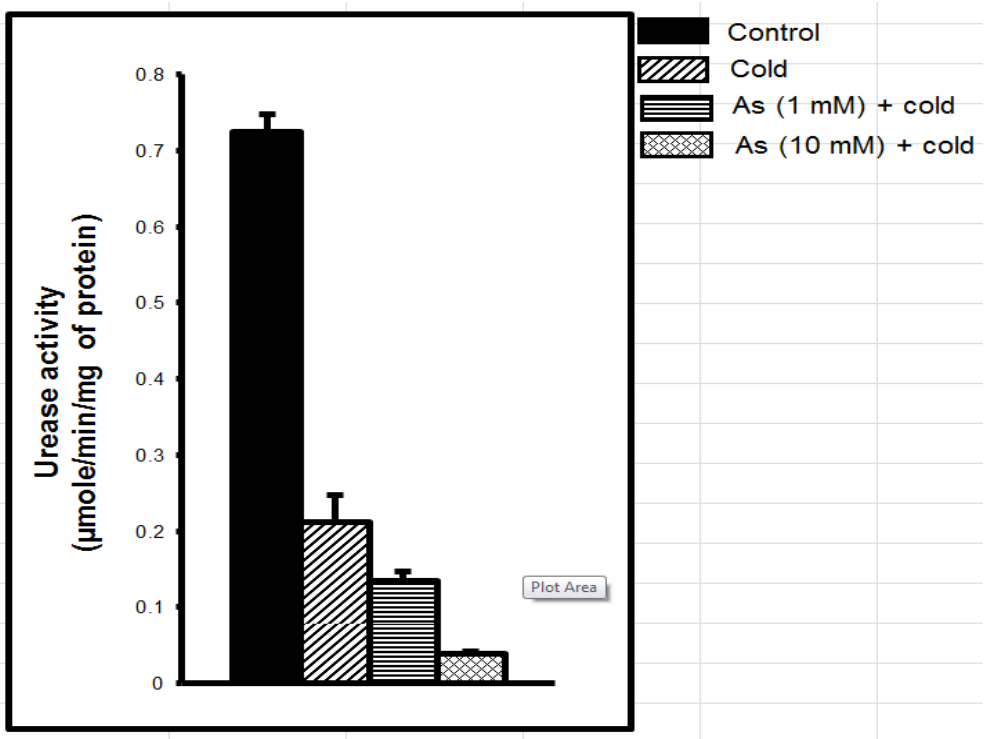

Figure 6. Effect of cold and Na_2HAsO_4 on urease activity in roots after 72h of treatment. The paddy was treated with different concentrations of arsenic (1 and 10 mM) and kept for 72h in the cold. The paddies in another pot were exposed to cold for 72h only in the cold chamber. Control paddy was similarly used without cold exposure and arsenic treatment. The data are means ± SE for 3 individual measurements in each group.

Therefore, the concentration of urea in lakes or natural soils is usually low and ranges between 0.1-3.0 µM (Cho *et al*., 1996), but upto 70 µM in fertilized crop-planted soils. With regard to this very low concentration it was believed that plants take up urea-derived nitrogen mainly in the form of ammonium (Polacco and Holland, 1993). We found that cold acclimation along with Na_2HAsO_4 causes an adverse environment where plants survive for their growth; therefore, the impaired root growth associated with reduced urease activity is possible. Previous study revealed that in woody species, cold hardening of roots is determined by genotype, soil temperature, and moisture (Wildung *et al*., 1973). However, little information on root cold hardiness and development following freezing is available for winter cereals. It has been suggested that roots and the lower portions of the crown of cereals are more susceptible to freezing injury than the leaves and upper crown tissue (Olien and Marchetti, 1976). There was a reduction in shoot and root growth in wheat plants frozen from -10 to -20^{o}C when transplanted to soil (Chen *et al*., 1983). The reduction in shoot growth was probably due to the effect of the lower temperatures on root regeneration. The growth of roots can be influenced by temperature gradients (Fortin and Poff, 1990), mechanical impedance (Barley and Greacen, 1967), aeration (Cannell, 1977) and the roots of adjacent plants (Mahall and Callaway, 1991). A recent study (Li *et al*., 2007) revealed that at higher concentration, arsenic is toxic to most plants. It interferes with metabolic processes and inhibits plant growth and development through arsenic induced phytotoxicity (Marin *et al*., 1993). When plants are exposed to excess arsenic either in soil or in solution culture, they exhibit toxicity symptoms such as: inhibition of seed germination (Abedin and Meharg, 2002); decrease in plant height (Marin *et al*., 1992), reduction in root growth (Abedin and Meharg, 2002), and lower fruit and grain yield (Abedin *et al*., 2002; Kang *et al*., 1996). Both cold acclimation and arsenic cause similar adverse environment, therefore, uptake of nitrogen to the plants is prohibited, utilization of soil urea is also affected because of the lower activity of urease. In these circumstances, a strategy might be developed either by administration of urea or other N-fertilizer. It is, therefore, the regulation of urease activity could be an index for the enhancement of the development of paddy growth.

4. Conclusion

Cold acclimation and arsenic have been found to be involved in the impairment of root development and plant growth. Although several factors might be involved in this respect, however, these two environmental and chemical effectors are found to be predominantly involved. The regulation of these two enzymes in response to cold and arsenic is an important aspect in plant metabolism and might be an index for the species of paddy for their growth during these critical environmental circumstances. Our investigations gave a concept to find a strategy for the survival of this plant species in these critical situations. It is assumed from the results that the impairment of root growth is mediated by the combined effects of these stressful effectors and coordinately to each other.

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