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Exogenous proline enhances the sensitivity of Tobacco BY-2 cells to arsenate

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Arsenic causes physiological and structural disorders in plants. Proline is accumulated as a compatible solute in plants under various stress conditions and mitigates stresses. Here, we investigated the effects of exogenous proline on tobacco Bright Yellow-2 (BY-2) cultured cells under AsO_4^- stress. Arsenate did not inhibit BY-2 cell growth at 40 and 50 μM but did it at 60 μM. Proline at 0.5 to 10 mM did not affect the cell growth but delayed it at 20 mM. At 40 μM AsO_4^-, neither 0.5 mM nor 1 mM proline affected the cell growth but 10 mM proline inhibited it. In the presence of AsO_4^-, 10 mM proline increased the number of Evans Blue-stained (dead) cells and decreased the number of total cells. Together, our results suggest that exogenous proline does not alleviate arsenate toxicity but enhances the sensitivity of BY-2 cells to arsenate.

Key words: arsenic; proline; cell growth; cell death; cell number

Arsenic, a toxic metalloid, is widely distributed in soil environment and causes physiological and structural disorders in plants.[1)] Arsenic accelerates cell death and inhibits plant growth.[2,3)] Nowadays, the reduction of crop yield by arsenic stress has been recognized as a threat to the sustainable food production.[4,5)] Arsenic occurs predominantly as inorganic forms such as arsenate (AsO_4^-) and arsenite (AsO_3^-). Plants take up arsenic mainly as arsenate but not arsenite.[6)]

The organic compatible solute, proline, has been reported to accumulate in plants subjected to various abiotic stresses such as salt stress.[7)] Exogenous proline functions as a free radical scavenger and an enzyme protectant.[8,9)] Okuma et al.[10)] reported that proline exhibits an antioxidant activity which was proved by the 1,1-diphenyl-2-picryldrazyl assay. Proline improves plant metabolism and stimulates plant growth under stress conditions.[11,12)] However, in some cases, exogenous proline showed its toxicity to plants[13)] and caused programmed cell death in plants.[14)]

In this study, we investigated the effects of exogenous proline on tobacco Bright Yellow-2 (BY-2) cultured cells under arsenate-stress conditions. We found that arsenate inhibited the BY-2 cell growth and that arsenate accelerated the inhibition of cell growth in the presence of proline. We also found that arsenate boosted the number of dead cells and decreased the total number of cells in the presence of proline. These results indicate that exogenous proline does not mitigate arsenate stress in BY-2 cells but that proline enhances the sensitivity of BY-2 cells to arsenate.

Materials and methods

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.[15,16)] The modified LS medium[17)] was used as a standard medium. The LS medium supplemented with 40, 50, and 60 μM AsO_4^- were regarded as the standard arsenic medium of 40, 50, and 60 μM AsO_4^-, respectively. The 0.5, 1, and 10 mM proline media were the 40 μM as well as 60 μM AsO_4^- media containing 0.5, 1, and 10 mM proline.

The BY-2 cells were cultured and maintained as described previously.[15,16)] The cells were subcultured weekly and were incubated on a rotary shaker at 100 rpm at 25 °C in the dark.

Measurement of BY-2 cell growth. The growth of BY-2 cells was measured as described previously.[15,16)] 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

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Abbreviations: BY-2, Bright Yellow-2; FW, fresh weight; DW, dry weight; DAI, days after inoculation; GSA, glutamate-5-semialdehyde; P5C, pyrroline-5-carboxylate; P5CDH, pyrroline-5-carboxylate dehydrogenase

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).

Estimation of cell death of BY-2 cells. Dead cells were quantified by the method described previously.[18,19] 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 four-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.

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

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.

Results

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 0.5, 1, 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 4–6 DAI and then steadily increased up to 10 DAI (Fig. 1(A) and (B)).

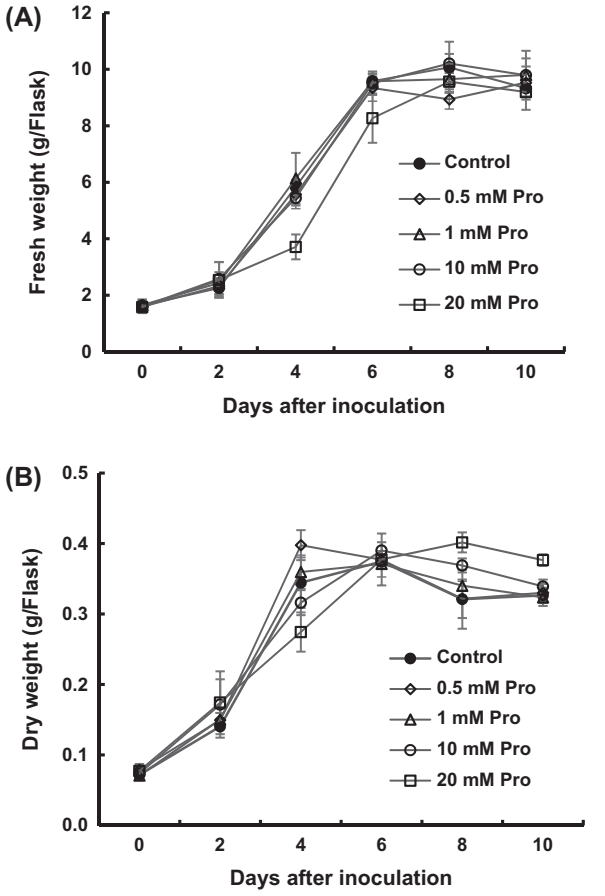

Fig. 1. Effect of exogenous proline (Pro) on BY-2 cell growth. (A) Shows the cell growth based on fresh weight and (B) shows the cell growth based on dry weight in response to 0.5, 1, 10, and 20 mM Pro at 0, 2, 4, 6, 8, and 10 days after inoculation. Averages of cell growth from three independent experiments ($n = 3$) 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 fresh weight or dry weight at each time point after inoculation.

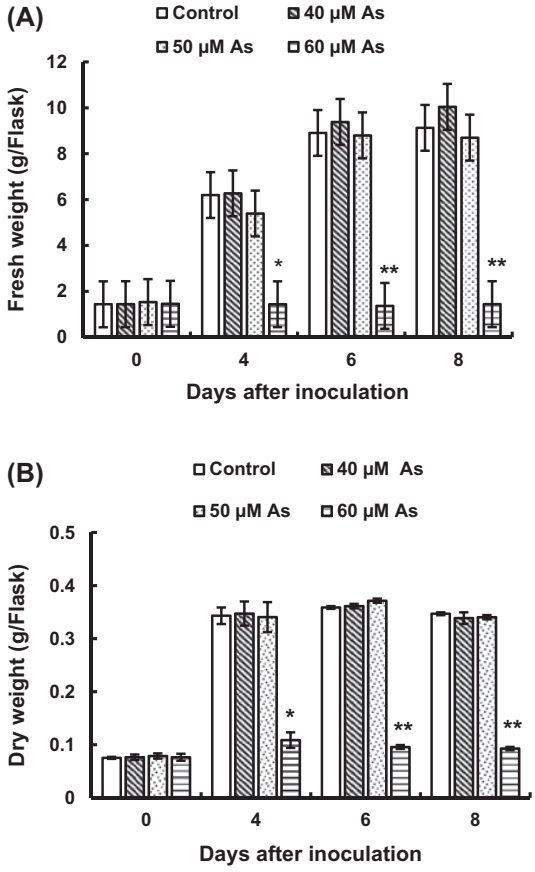

Fig. 2. Effect of exogenous arsenate (As) on BY-2 cell growth. (A) Shows the cell growth based on fresh weight and (B) shows the cell growth based on dry weight in response to 40, 50, and 60 µM arsenate at 0, 4, 6, and 8 days after inoculation.

Notes: Averages of cell growth from three independent experiments ($n = 3$) are shown. Error bars represent SE. * and **indicate $p < 0.05$ and $p < 0.01$, respectively as assessed by t-test.

Effects of exogenous arsenate on BY-2 cell growth

We tested the effects of exogenous 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, 50, and 60 μM AsO_4^-. Compared with control, arsenate did not affect the growth of BY-2 cells at 40 and 50 μM but significantly inhibited it at 60 μM at all DAI (Fig. 2(A) and (B)).

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.

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. 3(A) and (B)). At 60 μM AsO_4^- stress condition, 0.5 mM proline did not affect the cell growth but 1 mM and 10 mM proline significantly inhibited it (Fig. 4(A) and (B)). Moreover, AsO_4^- at 60 μM induced more inhibition of cell growth in the presence of exogenous proline than in the absence of exogenous proline. These results suggest that exogenous proline does not mitigate the arsenate-induced growth inhibition of BY-2 cells but enhances the sensitivity of BY-2 cells to arsenate.

Effects of exogenous proline on arsenate-induced cell death

We examined the cell death of BY-2 by arsenate in the presence and absence of proline. 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 and 1 mM proline (Fig. 5(A)).

Arsenate at 60 μM increased the number of Evans Blue-stained cells by 25% compared with control and that 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. 5(B)). These results indicate that application of proline does not recover the arsenate-induced cell death but increases the number of the dead cell by arsenate.

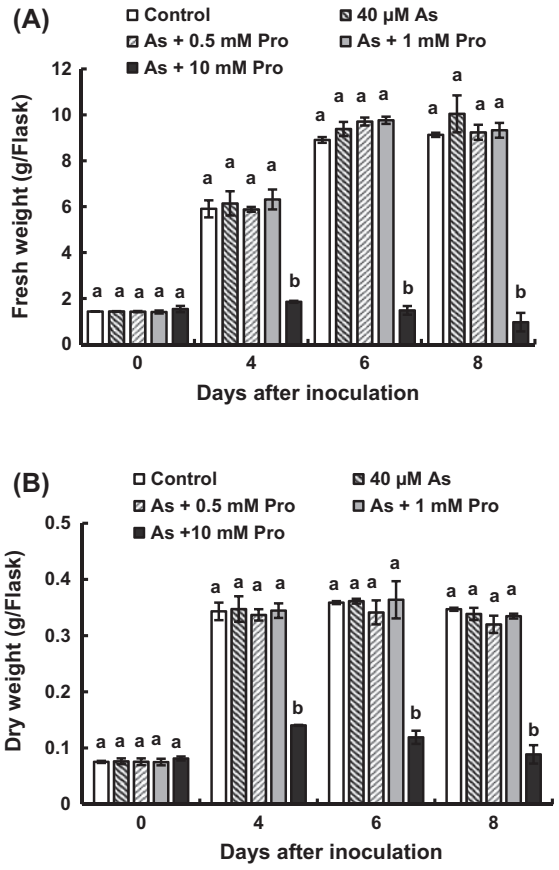

Fig. 3. Effect of exogenous proline (Pro) on 40 μM arsenate (As)-stressed BY-2 cells. Reduction of BY-2 cell growth by arsenate (Fresh weight basis, (A) dry weight basis, (B) in the presence of 10 mM Pro but not in the presence of 0.5 mM or 1 mM Pro at 4, 6, and 8 days after inoculation.

Notes: 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.

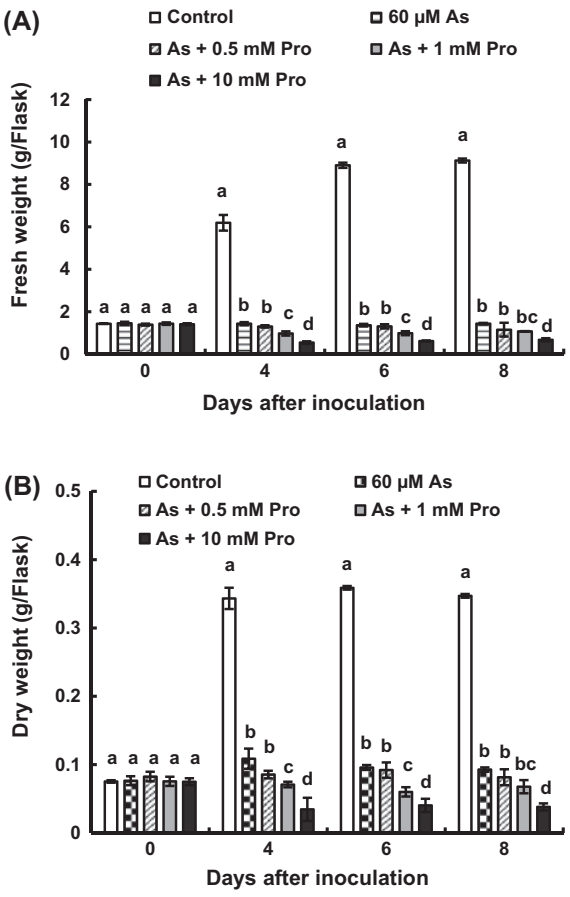

Fig. 4. Effect of exogenous proline (Pro) on 60 μM arsenate (As)-induced growth inhibition of BY-2 cells. Enhancement of arsenate-induced cell growth reduction (Fresh weight basis, (A) dry weight basis, (B)) in the presence of both the 1 and 10 mM Pro but not in the presence of 0.5 mM Pro at 4, 6, and 8 days after inoculation.

Note: See legend of Fig. 3 for an explanation of symbols.

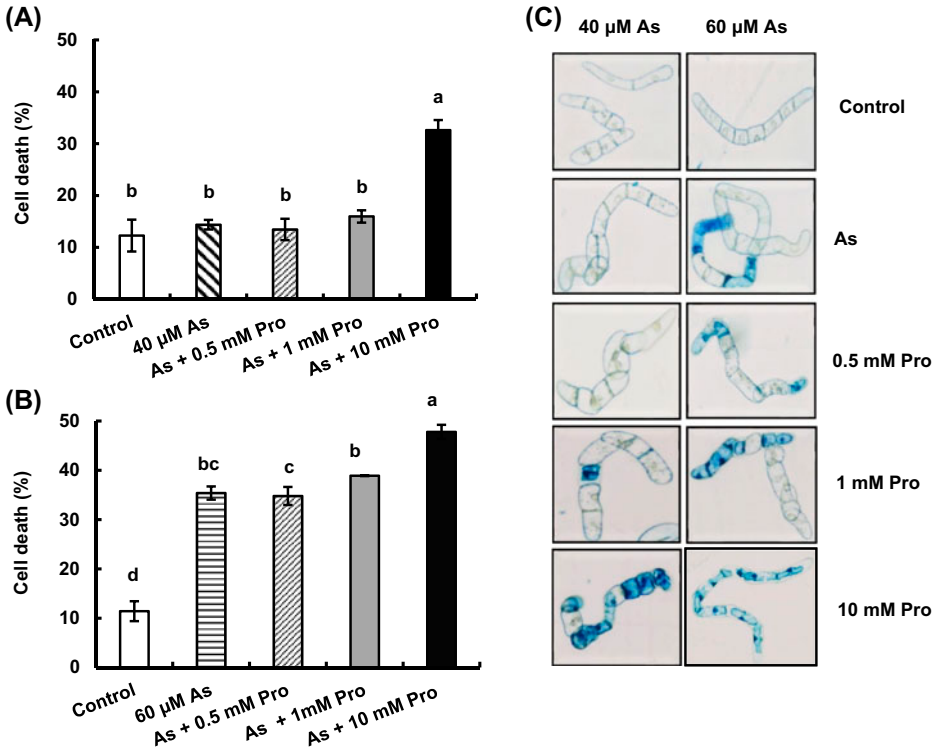

Fig. 5. Enhancement of arsenate (As)-induced BY-2 cell death by the application of proline (Pro). (A) Induction of cell death by the co-treatment of 40 μM arsenate and 10 mM Pro but not by the individual treatment of 40 μM arsenate. (B) Arsenate at 60 μM induced significant cell death which was enhanced in the presence of 10 mM Pro. 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) Evans Blue staining of arsenate-treated BY-2 cells in the presence or absence of Pro. At 40 μM arsenate-stress condition, BY-2 cells showed Evans Blue-stained cells in the presence of Pro but not in the absence. At 60 μM arsenate-stress condition, BY-2 cells showed more Evans Blue positive cells compared with control and that was enhanced in the presence of 10 mM Pro.

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

We monitored the effects of exogenous arsenate at 40 and 60 μM on the total number of BY-2 cells with or without the application of proline. We 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 and 1 mM proline (Fig. 6(A)).

We also found that arsenate at 60 μM decreased the total number of cells by 3.5-fold compared with control and that arsenate in the presence of 1 and 10 mM proline decreased the number of cells by 4.5- and 7.5-fold, respectively (Fig. 6(B)). These results indicate that application of proline does not recover the reduction of cell number by arsenate but enhances the arsenate-induced decrease of cells number.

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.[1,20] 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 and the increasing rate of growth inhibition by that arsenate in the presence of exogenous proline. We also demonstrate the AsO_4^--induced increment of cell death and the reduction of total cell number in the presence of exogenous proline, and thus, we elucidate the role of proline in BY-2 cells under arsenate stress.

It is well-known that arsenate inhibits the growth of plants.[21,22] Here, 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). 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 did not recover the arsenate-induced growth reduction but surprisingly that arsenate enhances the cell growth reduction in the presence of proline (Fig. 4). To insight into this issue, we further investigated the effects of 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. 3), suggesting that application of proline enhances the adverse effects of arsenate. 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 (Fig. 7). Arsenate is converted to arsenite by arsenate reductase and arsenite can act as an inhibitor of pyrroline-5-carboxylate dehydrogenase (P5CDH),[23,24]

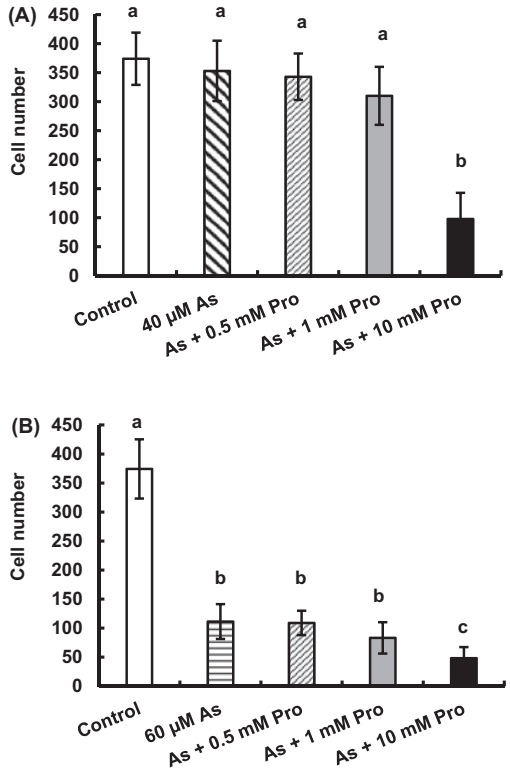

Fig. 6. Reduction of BY-2 cell number by arsenate (As) in the presence or absence of proline (Pro). (A) Decrease of cell number by the co-treatment of 40 μM arsenate and 10 mM Pro but not by the arsenate alone. (B) The reduction of cell number by the co-treatment of 60 μM arsenate and 10 mM Pro is higher than that of by the individual treatment of arsenate.

Notes: Averages of cell number from three independent experiments ($n = 3$) are shown. See legend of Fig. 5 for an explanation of symbols.

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 and then GSA/P5C is converted to glutamate by P5CDH.[14] 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.

In contrast to our results, Singh et al.[25] 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.[26] and Siddiqui et al.[27] 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 the present study, arsenate inhibited cell growth at 60 μM but not at 50 μM (Fig. 2). The drastic difference may be accounted for by combinational effects of endogenous proline that is converted to GSA/P5C and exogenous arsenate that inhibits P5CDH.

In this study, we found that arsenate induced the number of Evans Blue-stained (dead) cells (Fig. 5(B)) and decreased the total number of BY-2 cells (Fig. 6(B)), which are consistent with the previous results.[28] Moreover, we found that arsenate-induced cell death is boosted in the presence of proline, as well as the reduction of cell number by arsenate is potentiated in response to exogenous proline. These results suggest that arsenate increases the number of cell death and enhances the reduction of total cell number in the presence of 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

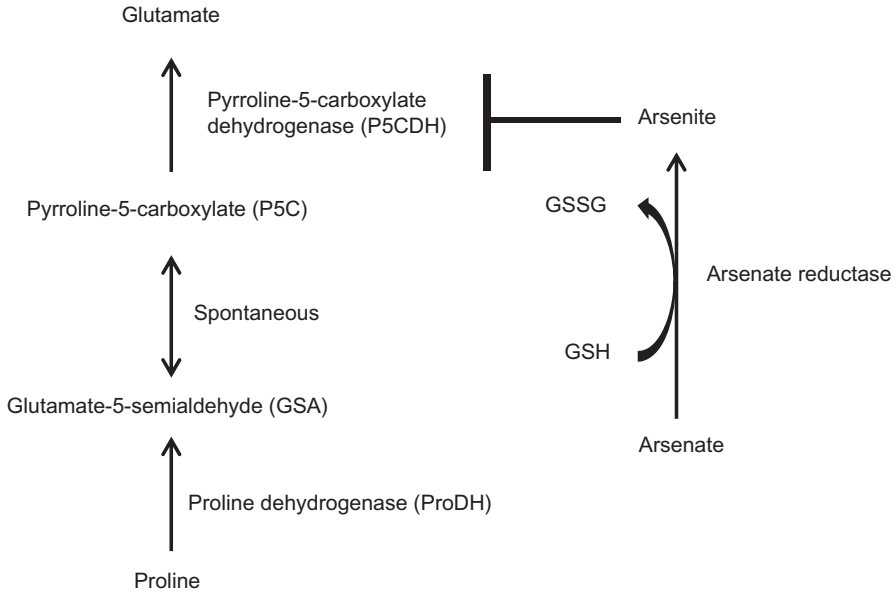

Fig. 7. A hypothetical flow diagram showing the inhibition of proline catabolic process by arsenite. Proline is degraded to glutamate catabolizing by the central enzymes ProDH and P5CDH. Arsenite inhibits P5CDH activity and hence accumulates P5C/GSA which is toxic to cells. GSH, reduced glutathione; GSSG, oxidized glutathione.

salt stress[29] and cadmium stress in BY-2 cells.[30] However, the negative role of exogenous proline was also reported in some cases. For instances, exogenous proline shows toxicity to plants[13,14] and proline at 2 mM inhibits the growth of saltgrass (*Distichlis spicata*).[31] 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[32] and did not show any protective value.[33] In the present study, our data showed that proline does not mitigate arsenic stress in BY-2 cells but arsenate induced more stressing effects in the presence of 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. Therefore, it can be said that proline is not a suitable osmoticum for the mitigation of arsenic stress in BY-2 cells.

Together, we conclude that exogenous proline does not mitigate arsenate stress but enhances the sensitivity of BY-2 cells to AsO_4^-.

Author contributions

M.N.N.N., A.Y. and M.A.H. performed experiments; M.M.I. and T. N. assisted in experiments and data analysis; S.M. and Y.N. contributed to the discussion; M.N.N.N., M.Y.P., and Y.M. wrote the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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