


Exogenous proline enhances antioxidant enzyme activities but does not mitigate growth inhibition by selenate stress in tobacco BY-2 cells

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ABSTRACT

Selenium (Se) causes oxidative damage to plants. Proline is accumulated as a compatible solute in plants under stress conditions and mitigates stresses. Selenate at 250 μM increased cell death and inhibited the growth of tobacco BY-2 cells while exogenous proline at 10 mM did not mitigate the inhibition by selenate. Selenate increased accumulation of Se and ROS and activities of antioxidant enzymes but not lipid peroxidation in the BY-2 cells. Proline increased Se accumulation and antioxidant enzyme activities but not either ROS accumulation or lipid peroxidation in the selenate-stressed cells. Glutathione (GSH) rather than ascorbic acid (AsA) mitigated the growth inhibition although both reduced the accumulation of ROS induced by selenate. These results indicate that proline increases both antioxidant enzyme activities and Se accumulation, which overall fails to ameliorate the growth inhibition by selenate and that the growth inhibition is not accounted for only by ROS accumulation.

Abbreviations: APX: ascorbate peroxidase; AsA: ascorbic acid; BY-2: Bright Yellow-2; CAT: catalase; DAL: days after inoculation; DW: dry weight; FW: fresh weight; GSH: glutathione; ROS: reactive oxygen species

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Selenium (Se) is a micro-nutrient for animals but not for most plants [1,2] and Se concentrations at higher than 100 μM can be hazardous for all biotas including plants and animals [3]. In human, adequate level of Se has an important role in antioxidant selenoproteins for protection against oxidative stress while high dose of Se shows alkali diseases and disorders of nervous system [4,5]. In plants, lower concentration of Se has been shown to have positive effects on stress tolerance. For example, exogenous application of selenium alleviates cadmium-induced oxidative stress in tomato (*Solanum lycopersicum* L.) plants by enhancing antioxidant system [6] and salinity-induced damage in rapeseed (*Brassica napus*) seedlings [7]. In contrast, high concentration (>100 μM) of Se is toxic for plants as well as animals [3,8].

Selenium toxicity occurs in plants for its chemical similarity with sulfur. Substitution of cysteine residues and methionine residue by selenocystein residue and selenomethionine residue adversely affects the activities of proteins, which result in mimic sulfur starvation such as withering, drying, and premature death of leaves [9–11]. Therefore, it is crucial to understand the mechanisms of growth inhibition by selenium in plants in order to develop approaches to alleviate Se stress.

Proline is a bioactive molecule that functions as an osmoprotectant. It is well known that proline accumulates in plants during various stresses [12–15]. Heavy

metal toxicity and plant–pathogen interaction also cause proline accumulation in plants [16,17]. Supplementation of proline can improve tolerance to a variety of environmental stresses in plants [18]. In the case of tobacco Bright Yellow-2 (BY-2) cells, the growth inhibition by salt or cadmium (Cd) was alleviated by supplementation of proline at 10 mM [19–22]. It has been stated that proline plays a role as an enzyme protectant and reactive oxygen species (ROS) scavengers [20,23–25]. However, growth inhibition by arsenate was alleviated by proline only at lower concentrations in BY-2 cells [26].

A variety of toxic metals can induce ROS accumulation in the plant tissues. The ROS accumulation is responsible for damaging biological molecules such as proteins, lipids, DNA, and carbohydrates [27–31]. Selenium functions as a pro-oxidant at high doses, that is, selenium induces ROS production, which triggers oxidative stress in plants. In addition, ROS also acts as a key signaling molecule to mediate many physiological processes in plants such as metabolism, growth, and development, and response to different stress [32,33].

Plants have enzymatic and non-enzymatic antioxidant defense systems, which play an important role to detoxify the produced ROS. In plant cells, ascorbic acid (AsA) is a key non-enzymatic antioxidant that can directly scavenge ROS such as H_2O_2 , $\text{O}_2^{\cdot-}$, OH^{\cdot} ,

and $^1\text{O}_2$ and plays an important role in ascorbate-glutathione (AsA-GSH) cycle for scavenging H_2O_2 . Glutathione (GSH) is another important non-enzymatic antioxidant in plant cells that also either directly or indirectly scavenges a range of ROS such as H_2O_2 , $\text{OH}\cdot$, and $^1\text{O}_2$ [27,34,35]. In the AsA-GSH cycle, ascorbate peroxidase oxidizes AsA to produce monodehydroascorbate (MDHA) to detoxify H_2O_2 and monodehydroascorbate reductase (MDAR) mediates regeneration of AsA from MDHA using NADH. The MDHA can disproportionate into ascorbate and dehydroascorbate (DHA) when MDHA is not immediately reduced to AsA. Dehydroascorbate reductase (DHAR) reduces DHA to AsA using reduced GSH. The subsequent oxidized GSH (GSSG) is converted to reduced GSH by NADPH-dependent glutathione reductase (GR).

Tobacco is an annual crop, which is commercially cultivated in many countries and tobacco BY-2 cells have been widely used in many biochemical studies because they are highly homogenous and can grow rapidly in suitable culture medium. Exogenous proline reduces intracellular ROS accumulation and oxidative damage by increasing antioxidant enzyme activities in tobacco BY-2 cells under Na and Cd stress [21,22]. Moreover, in some cases, the exogenously applied proline is toxic to plants [36]. However, it remains to be clarified whether the exogenous application of proline can mitigate inhibition of growth by selenate in BY-2 cells. Therefore, the effects of exogenous proline on growth, antioxidant enzyme activities, intracellular ROS accumulation, and level of oxidative damage in tobacco BY-2 cultured cells under selenate stress were investigated.

Materials and methods

Cell cultures of tobacco BY-2

Tobacco (*Nicotiana tabacum* L., cv. BY-2) suspension-cultured cells were employed as a cell line that is unadapted to selenium stress [37,38]. The modified LS medium [39] was used as a control medium. Selenium medium was prepared by supplementation with $250\text{ }\mu\text{M}$ Na_2SeO_4 in the control medium. In order to examine proline effects, the control medium and the selenium medium supplemented with 5 mM or 10 mM proline were used. The cell cultures were performed and managed, as previously described [37,38].

Assay of BY-2 cell growth

Two-gram cells were collected from 7 days old suspension culture and inoculated in fresh medium. The medium was cultured on a rotary shaker (100 rpm , 25°C). The fresh weight (FW) of BY-2 was measured at 0, 4, 6, and 8 days after inoculation as described

previously [37,38]. The fresh BY-2 cells were oven-dried at 70°C and the dry weight (DW) was measured after 24 h.

Estimation of cell death of BY-2 cells

Dead cells were estimated by the following procedure of Nahar et al. [26]. Cells were stained with Evans Blue solution (0.05%) for 20 min to load the dye. After that, the excess dye was washed out with distilled water. Dye-bounded dead cells were 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 2-day-old and 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.

Intracellular ROS detections

Intracellular ROS accumulation in BY-2 cells was analyzed by using $2',7'$ -dichlorodihydrofluorescein diacetate ($\text{H}_2\text{DCF-DA}$) as described previously [40]. Four-day-old BY-2 cells were collected with brief centrifugation by washing twice with phosphate-buffered saline (PBS). Then the collected cells were incubated in PBS supplemented with $20\text{ }\mu\text{M}$ $\text{H}_2\text{DCF-DA}$ for 30 min at 30°C . After that the incubated cells were washed two times with PBS and fluorescence images were captured using a fluorescence microscope (Biozero, Keyence, Japan) and analyzed using Image-J software (NIH, USA).

Determination of lipid peroxidation

Lipid peroxidation was evaluated by measuring malondialdehyde (MDA) contents, as previously described by Okuma et al. [41]. One-gram of BY-2 cells was grounded in mortar and pestle using liquid nitrogen (N_2) and 2 mL of 5% (w/v) trichloroacetic acid was added to homogenize the mixture. Then the homogenized mixture was centrifuged at $12,000\text{ g}$ for 15 min. After that, the supernatant was collected and diluted twice with 20% (w/v) trichloroacetic acid containing 0.5% (w/v) thiobarbituric acid. Then the mixture was heated at 95°C for 25 min. After cooled in an ice bath, the mixture was centrifuged at 7500 g for 5 min. At 532 nm , the absorbance of the samples was measured and nonspecific turbidity was compensated by subtracting absorbance at 600 nm . MDA content was calculated using extinction of the coefficient of $155\text{ mM}^{-1}\text{cm}^{-1}$.

Measurement of antioxidant enzyme activities

Catalase activity

Catalase (CAT; EC: 1.11.1.16) activity was analyzed by the following procedure of Islam et al. [22]. A reaction mixture was prepared which contained 50 mM Tris-HCl buffer (pH 8.0), 0.25 mM EDTA, 20 mM H₂O₂, and 25 µL of the sample solution. The catalase activity was calculated from the decrease in absorbance at 240 nm for 120 s when the extinction coefficient was 40 M⁻¹ cm⁻¹.

APX activity

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined by the following procedure of Ishikawa et al. [42]. The reaction buffer solution contained 50 mM KH₂PO₄ buffer (pH 7.0), 0.1 mM EDTA, 0.1 mM H₂O₂, and 0.5 mM AsA. The reaction was started by adding the sample solution to the reaction buffer solution. The oxidation of ascorbate was followed by a decrease in absorbance at 290 nm for 60 s, where the extinction coefficient was 2.80 mM⁻¹ cm⁻¹.

SOD activity

Superoxide dismutase (SOD) activity was measured by using a SOD Assay Kit-WST (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). For each SOD activity measurement, 20 µL of the sample solution was added to the wells for sample and blank 2. Twenty microliters of distilled water was added in the wells for blanks 1 and 3. Two hundred microliters of WST working solution was added to each well and mixed properly. Then, 20 µL of dilution buffer was added to the wells for blanks 2 and 3. After that, 20 µL of enzyme working solution was added to the wells for sample and blank 1 and then mixed thoroughly. The absorbance was measured at 450 nm using a microplate reader after incubation at 37°C for 20 min. The SOD activity was calculated (inhibition rate %) using the following equation: SOD activity (inhibition rate %) = [(A blank 1-A blank 3)-(A sample-A blank 2)]/(A blank 1-A blank 3)×100.

POX activity

Peroxidase (POX) activity was analyzed by the following procedure of Hoque et al. [21]. A reaction buffer solution was prepared which contained 50 mM KH₂PO₄ buffer (pH 7.0), 0.1 mM EDTA, 0.1 mM H₂O₂ and 10 mM guaiacol. The reaction was started by the addition of the sample solution to the reaction buffer solution. The activity was calculated from change in absorbance at 470 nm for 30 s where an extinction coefficient is 26.6 mM M⁻¹ cm⁻¹.

Determination of Se contents

Selenium contents were determined by the following procedure of Ohno et al. [43] with modifications. Each sample was digested with 5 mL of HNO₃ at 130–150°C

and then treated with 1 mL of concentrated HCl. The Se was extracted with distilled water, and the amounts of extracted Se were quantified by fluorometric assay using 2,3-diaminonaphthalene (DAN). Concisely, 2 mL of masking reagent (EDTA, NH₄OH, NH₂OH.HCl, and methyl orange) was added to the cooled extraction. The volume mixture was adjusted to 9 mL, and then freshly prepared 0.1% DAN (in 0.1 N HCl) was added in the mixture and incubate it 30 min at 50°C. After cooled the mixture, 5 mL of cyclohexane was added to the mixture and the fluorescence of the cyclohexane solution was measured at 520 nm (excitation, 375 nm) using a fluorescence spectrophotometer. Selenium standard solution (Wako, Japan) was used to measure the Se contents.

Measurement of protein content

Protein contents were determined as described previously [44].

Statistical analysis

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

Exogenous proline did not mitigate growth inhibition of BY-2 cells under selenate stress

In order to investigate effects of selenate and proline on cell growth of BY-2 cells, we tested 250 µM selenate (Figure 1), which is toxic but not severely to rice plants [8], and 10 mM proline, which improves salt tolerance and cadmium tolerance in BY-2 cells [20–22].

The effects of selenate on BY-2 cell growth were examined by measuring the fresh weight (FW) and dry weight (DW) of selenate-stressed BY-2 cells at 0, 4, 6, and 8 days after inoculation (DAI). The FW of BY-2 cells cultured in the selenium medium was significantly lower than that in the control medium at 4, 6, and 8 DAI (Figure 1(a)). The DW of BY-2 cultured cells in the selenium medium was significantly lower than that in the control medium at 4 and 6 DAI (Figure 1(b)). These results indicate that selenate at 250 µM significantly inhibits the growth of BY-2 cells.

In order to investigate whether exogenous proline mitigates the inhibitory effects of selenate on the growth of BY-2 cells, the FW and DW of BY-2 cells at 0, 4, 6, and 8 DAI were measured. Application of proline at 5 mM and 10 mM did not significantly ameliorate the selenate-induced growth inhibition in the BY-2 cells (Figure 1(a,b)), suggest that the

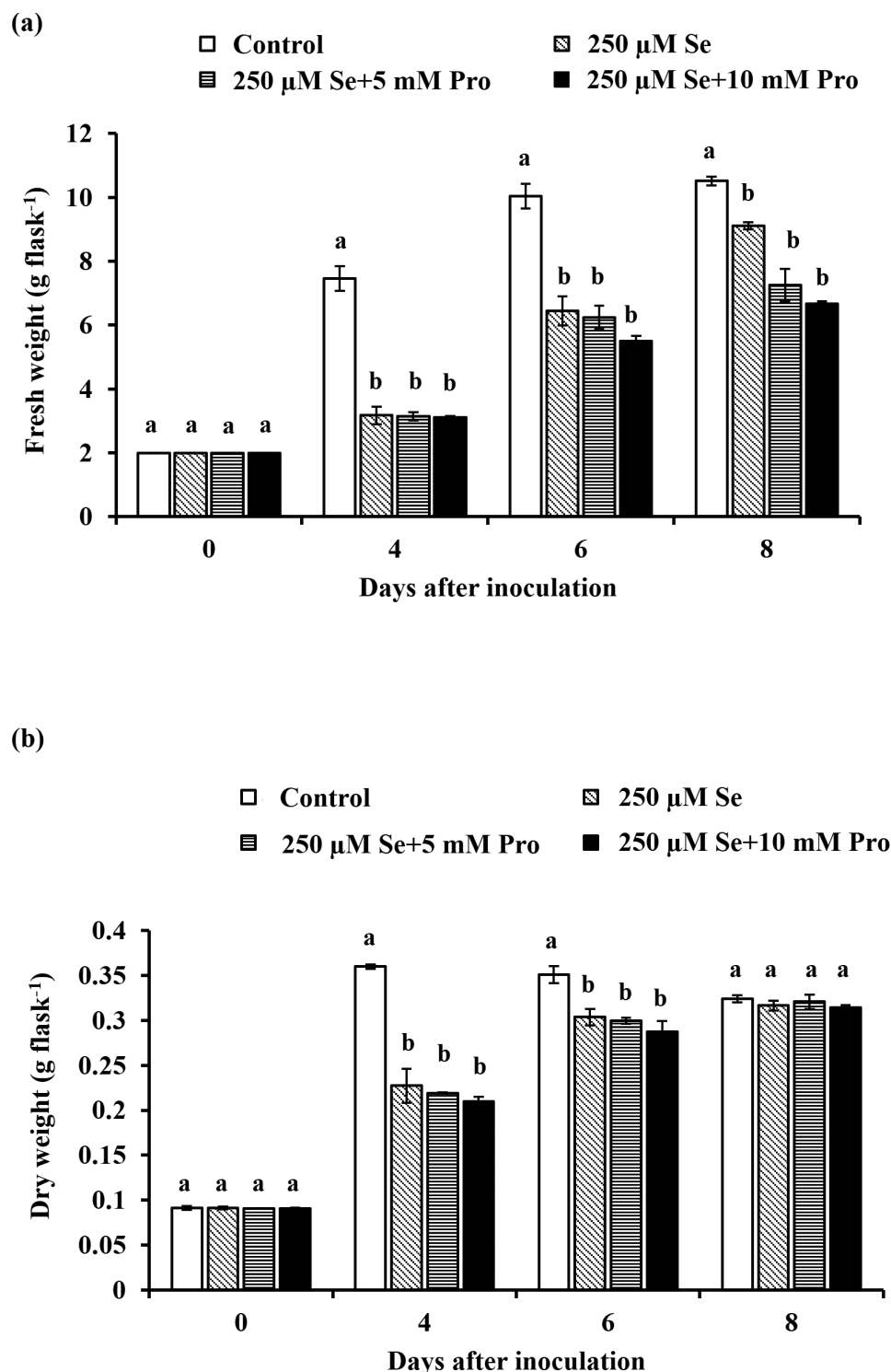


Figure 1. Effects of exogenous proline (Pro) on BY-2 cell growth under selenate stress. Effects of 5 mM and 10 mM proline on BY-2 cell growth at 0, 4, 6, and 8 days after inoculation (DAI) under 250 μ M selenate stress based on fresh weight (a) and (b) based on dry weight. Averages of cell growth from three independent experiments ($n = 3$) are shown. Error bars represent SE. For the same inoculation day, bars with the same letters are not significantly different at $P < 0.05$.

mechanism of growth inhibition by selenium is different from that by other metals such as Na and Cd.

Exogenously applied proline did not recover selenate-induced cell death

We used Evans Blue to evaluate the cell death of BY-2 induced by selenate in the presence and absence of

proline. Under non-stress control condition, the stained cells accounted for 2.8% and 8.8% in the absence of proline at 2 days and 4 days, respectively, and 4.9% and 9.5% in the presence of 10 mM proline at 2 days and 4 days, respectively (Figure 2(a)). When treated with selenate, the percentage of stained cells was increased by 10.7% and 28.6% in the absence of proline at 2 days and 4 days, respectively, and by 14.5%

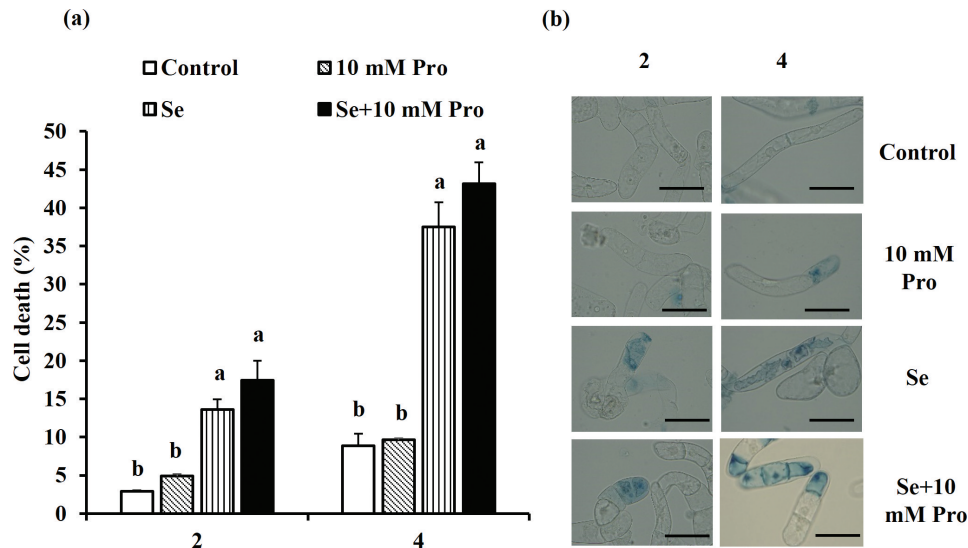


Figure 2. Effects of exogenous application of proline on selenate-induced BY-2 cell death. (a) Selenate at 250 μM significantly induced cell death and supplementation of proline at 10 mM did not recover the selenate-induced cell death. Averages of cell death from three independent experiments ($n = 3$) 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. (b) Representative images showing that Evans Blue staining of selenate-treated BY-2 cells in the presence or absence of proline. Scale bar = 20 μm .

and 34.2% in the presence of proline at 2 days and 4 days, respectively (Figure 2(a)). These results indicate that selenate drastically induces cell death of BY-2 but that application of proline does not affect the cell death.

Exogenously applied proline did not affect ROS accumulation in BY-2 cells under selenate stress

Intracellular ROS accumulation in the BY-2 cells was evaluated by using 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA). The application of 250 μM selenate significantly induced the accumulation of intracellular ROS in the BY-2 cells (Figure 3(a)). Exogenous proline at 10 mM did not affect the ROS accumulation induced by selenate stress (Figure 3(a)).

Effects of supplementation of proline on lipid peroxidation of BY-2 cells under selenate stress

Effects of supplementation of proline on lipid peroxidation of selenate-stressed BY-2 cells were investigated using thiobarbituric acid. Selenate at 250 μM did not increase the MDA contents irrespective of the presence or absence of 10 mM proline in the culture medium (Figure 3(c)), suggesting that selenate stress does not induce lipid peroxidation in the BY-2 cells.

Antioxidant enzyme activities of BY-2 cells under selenate stress

In order to investigate the mitigation effects of proline on the selenate-stressed plants, activities of antioxidant enzymes, APX, CAT, SOD, and POX in the BY-2 cells under selenate stress were examined. The

activities of APX, CAT, SOD, and POX were significantly increased in the BY-2 cells in response to selenate stress (Figure 4). Exogenous application of 10 mM proline significantly increased the APX and POX activities of the selenate-stressed cells (Figure 4(a,d)) but did not significantly increase the CAT or SOD activity of the stressed cells (Figure 4(b,c)). These results indicate that selenate stress increases antioxidant enzyme activities and that exogenous proline does not increase all antioxidant enzyme activities of BY-2 cells under selenate stress.

Exogenously applied proline enhanced the Se accumulation under selenate stress

To evaluate the effects of exogenous application of proline on Se accumulation, we determined Se contents in BY-2 cells under selenate stress in the presence or absence of proline. Selenate stress significantly increased Se contents in BY-2 cells (Figure 5) and exogenous application of 10 mM proline enhanced the Se accumulation in the selenate-stressed BY-2 cells (Figure 5).

Effects of ROS scavengers in BY-2 cells under selenate stress

Ascorbic acid (AsA) and glutathione (GSH) are non-enzymatic antioxidants, which are capable of scavenging ROS. To clarify whether these ROS scavengers mitigate the growth inhibition by selenate stress, the effects of GSH and AsA were examined in the BY-2 cells under selenate stress. Glutathione at 0.5 mM significantly mitigated the selenate-induced growth inhibition whereas AsA at 20 mM did not ameliorate the growth inhibition

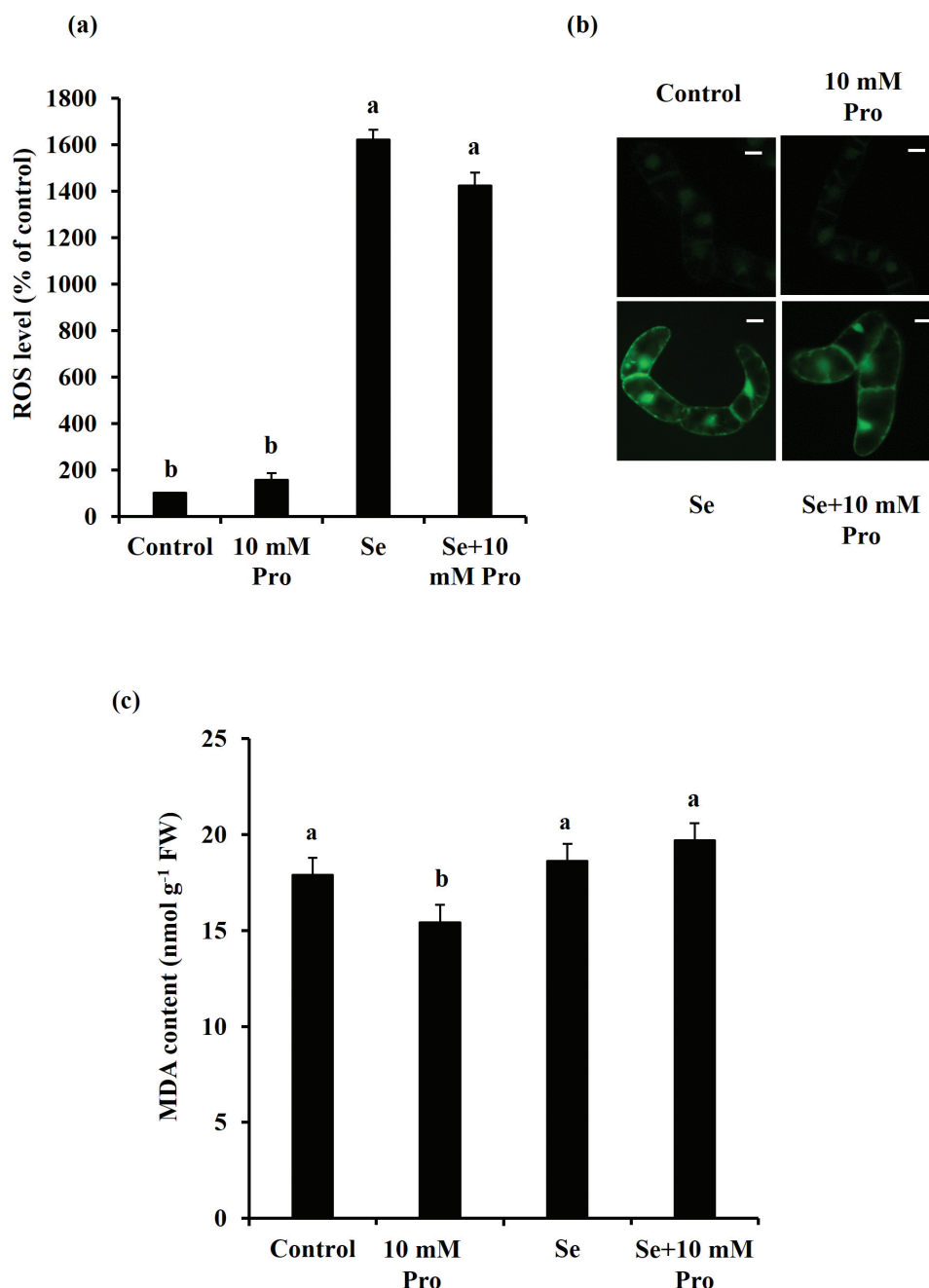


Figure 3. Effects of exogenous proline (Pro) on intracellular ROS accumulation and lipid peroxidation on BY-2 cells under selenate stress. (a) Intracellular ROS level in 4-day-old Se-unadapted tobacco BY-2 suspension cells under selenate stress in the presence or absence of proline. (b) Representative fluorescence images of 4-day-old Se-unadapted tobacco BY-2 suspension cells under selenate stress in the presence or absence of proline. Scale bar = 20 μ m. (c) Malondialdehyde (MDA) content in 4-day-old Se-unadapted tobacco BY-2 suspension cells under selenate stress in the presence or absence of proline. The percentage of fluorescence intensity is shown as a ROS level. Averages from three independent experiments ($n = 3$) per bar are shown. Error bars represent SE. Bars with the same letters are not significantly different at $P < 0.05$.

by selenate stress (Figure 6). Furthermore, the effects of AsA and GSH on intracellular ROS accumulation in the BY-2 cells under selenate stress were examined. Not only AsA at 20 mM but also GSH at 0.5 mM significantly decreased the intracellular ROS accumulation in the BY-2 cells under selenate stress, where neither AsA nor GSH changes the ROS accumulation in the non-stressed BY-2 cells (Figure 7). These results suggest that the growth inhibition by selenate stress is not accounted for by ROS accumulation.

Discussion

Many studies reported that proline plays a defensive role against various stresses in plants and cultured cells. Exogenous proline is known to alleviate Na- and Cd-induced growth inhibition in BY-2 cells [21,22,41] and exogenous proline at lower concentrations suppressed growth inhibition of BY-2 cells by arsenate stress [26]. However, the defensive role of proline against selenate stress in BY-2 cells remains to be understood. Selenate

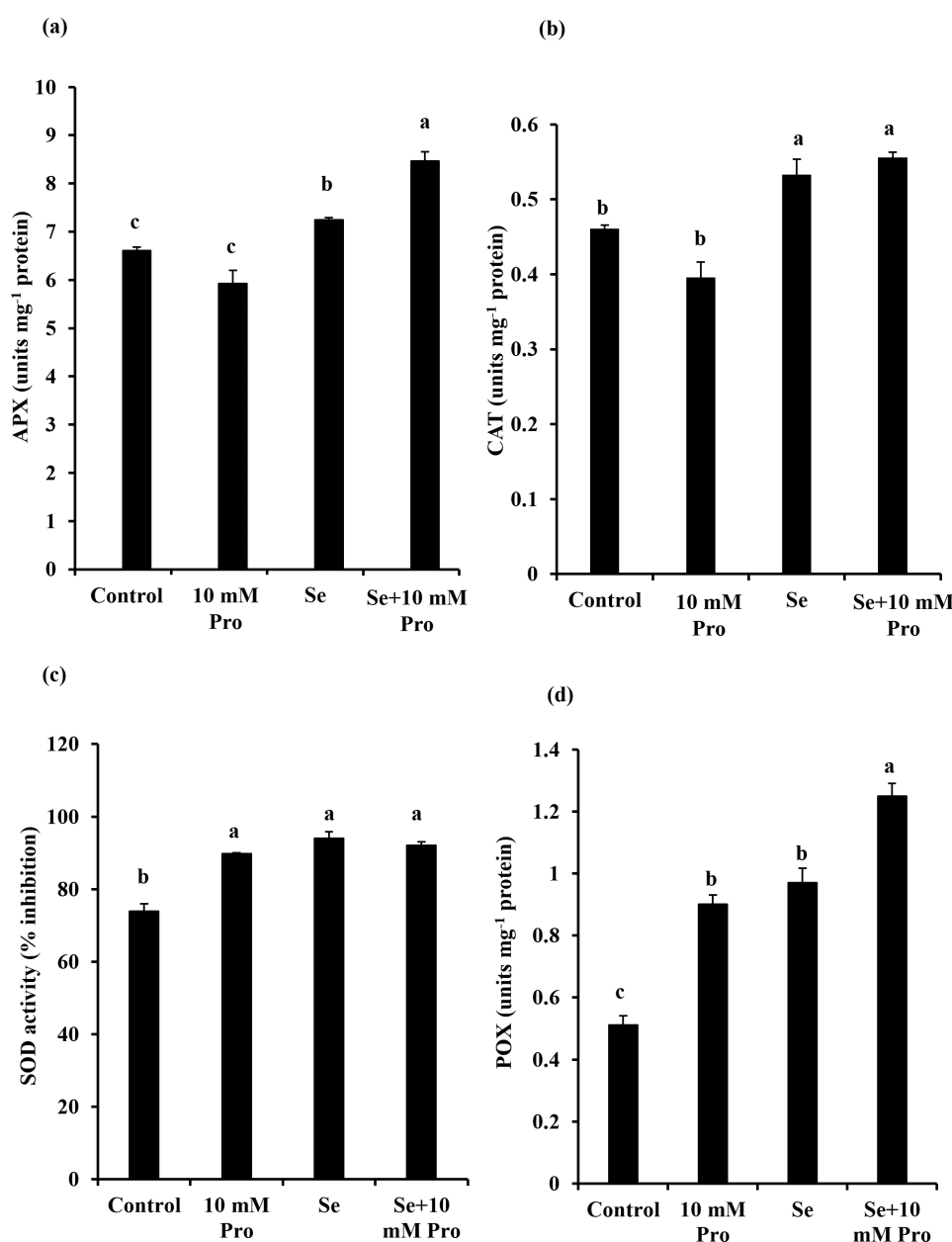


Figure 4. Activities of antioxidant enzymes (a) APX, (b) CAT, (c) SOD, and (d) POX in 4-day-old Se-unadapted tobacco BY-2 suspension cells induced by proline and selenate. The activity of APX, CAT, and POX was expressed in units mg⁻¹ protein and the SOD activity was expressed in % inhibition. One unit of activity was defined as the μmol substrate metabolized per min. Averages from three independent experiments ($n = 3$) per bar are shown. Error bars represent SE. Bars with the same letters are not significantly different at $P < 0.05$.

toxicity was also evident from the inhibition of growth in selenate-stressed BY-2 cells (Figure 1(a,b)) but proline did not mitigate the growth inhibition by selenate (Figure 1(a,b)). However, Aggarwal et al. [10] reported that exogenous proline alleviates phytotoxic effects of selenium by reducing oxidative stress and improves growth in Bean (*Phaseolus vulgaris* L.) seedlings. This difference may come from the difference in endogenous proline contents between BY-2 cells (approximately 3 mM) [26] and bean seedlings (approximately 2 μM) [45]. In addition, selenate stress may affect proline metabolism. Proline content has been known to be involved in salt, drought, and heavy-metal tolerance

mechanisms in plants [46–48]. Under stress conditions, the level of proline is maintained by biosynthetic enzyme pyrroline-5-carboxylate synthetase (P5CS) as well as degrading enzyme proline dehydrogenase (ProDH). 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 pyrroline-5-carboxylate dehydrogenase (P5CDH) [15,49,50]. Therefore, the reduction of cytotoxic GSA is critical to the stress mitigation by exogenous proline, that is, inhibition of P5CDH activity by the stress can impair the mitigation by proline. The present results in this study suggest that

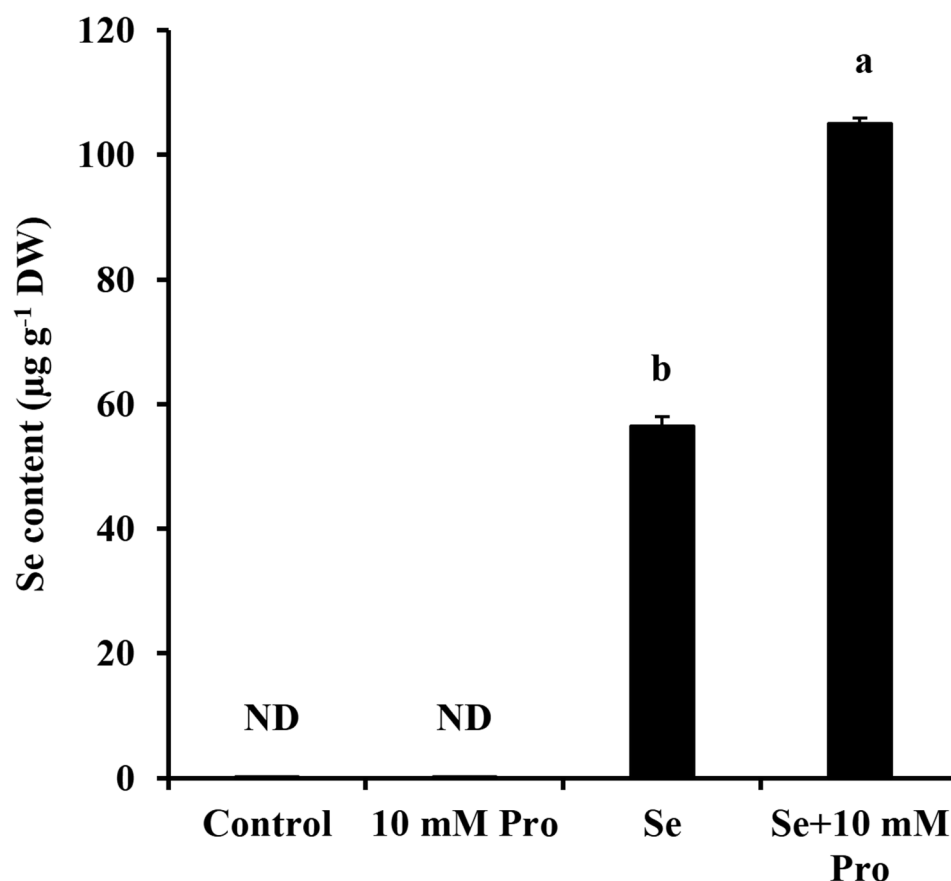


Figure 5. Effects of exogenous application of proline on Se accumulation. Selenium contents in 4-d-old Se-unadapted tobacco BY-2 suspension cells induced by proline under Se stress. Averages from three independent experiments ($n = 3$) per bar are shown. "ND" indicates the not detected. Error bars represent SE. Bars with the same letters are not significantly different at $P < 0.05$.

selenate inhibits P5CDH, resulting in accumulation of cytotoxic GSA. However, it is difficult to quantitate GSA and P5C because they are an equilibrated mixture.

Both salt stress and arsenate stress increased the number of Evans Blue-stained BY-2 cells [26,29]. However, the salt-induced cell death was suppressed by supplementation of proline [29] but the arsenate-induced cell death was aggravated by proline [26]. Proline induced cell death by ProDH activation, which leads to GSA accumulation [15]. Hence, the difference in response to proline between salt-induced cell death and arsenate-induced cell death may be due to proline metabolism such as GSA accumulation. In this study, selenate stress induced cell death of BY-2 but the cell death was not affected by exogenous proline (Figure 2), which may be accounted for the proline metabolism.

Several studies have reported that selenate stress can increase intracellular ROS accumulation in plants [51–53]. Similarly, this study showed that intracellular ROS accumulation was increased by selenate stress (Figure 3(a)). In plants, low concentration of Se reduces the toxic effects of heavy metals by alleviating oxidative damages while high dose of Se induces the oxidative stress [54–56]. In this study, selenate stress

increased antioxidant activities (Figure 4) and drastically induced selenate uptake (Figure 5), resulting in growth inhibition and cell death.

Islam et al. [22] reported that Cd stress increased lipid peroxidation and supplementation of proline decreased Cd-induced lipid peroxidation in BY-2 cells. Several studies have reported that salt-induced lipid peroxidation is decreased by the exogenous application of proline [29,41,57]. The present study showed that selenate stress did not affect the MDA content and that exogenous proline also did not show any effect on MDA content under selenate stress (Figure 3(c)). These results indicate that the level of oxidative damage was not high enough to induce lipid peroxidation in selenate-treated BY-2 cells and that selenate inhibited BY-2 cell growth not via increasing oxidative damage.

It is well known that proline plays an important role in protecting different enzymes against stresses such as salinity stress [20,23–25]. Some previous studies reported that the antioxidant defense system under salt and Cd stress was increased by exogenous proline application in BY-2 cells [21,29,41]. Hossain et al. [58] reported that the ascorbate peroxidase (APX) activity was increased in response to Cd stress on mung bean

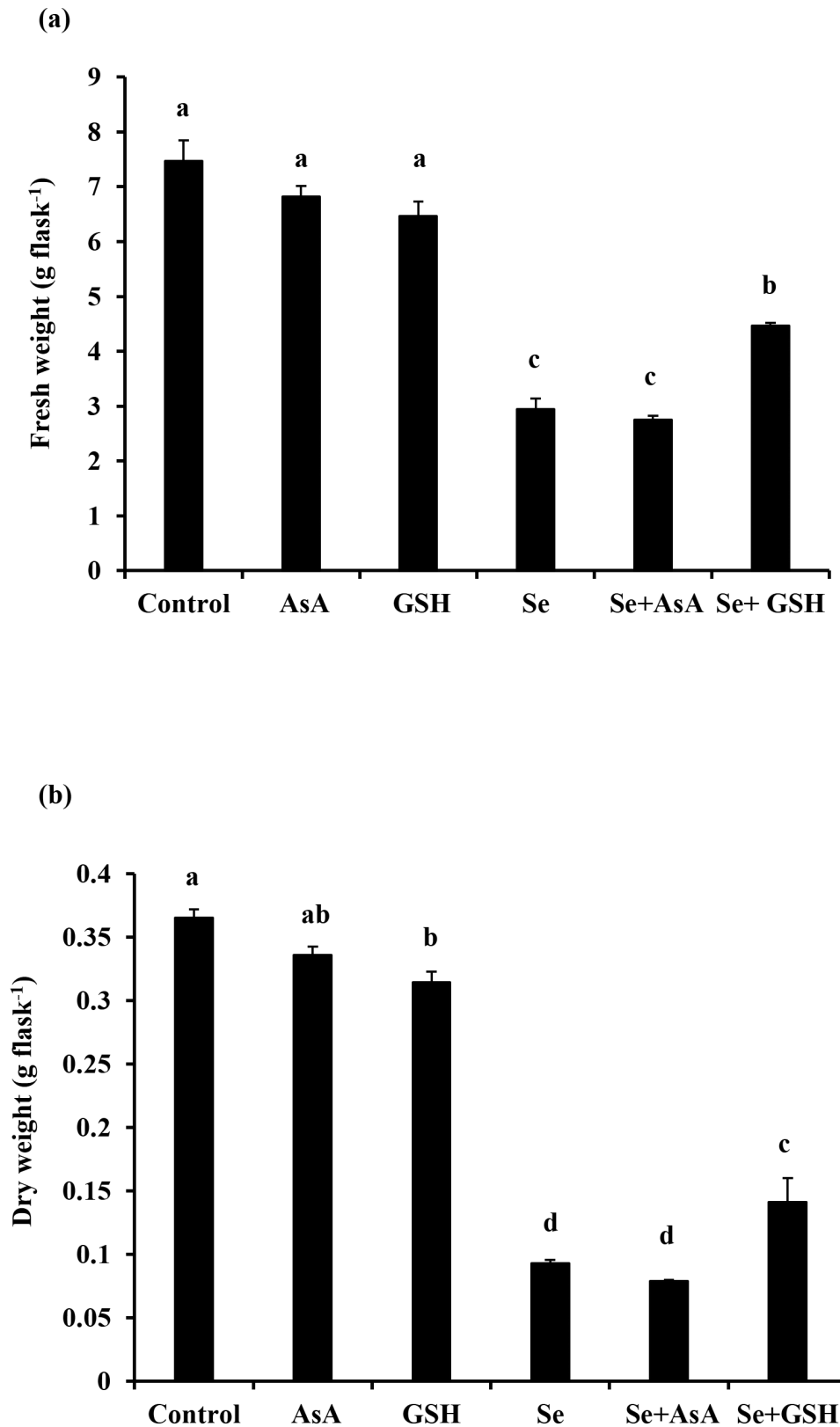


Figure 6. Effects of ascorbic acid (AsA) and glutathione (GSH) on 4-day-old BY-2 cell growth under selenate stress. (a) Shows the cell growth on the basis of fresh weight and (b) shows the cell growth on the basis dry weight in response to 20 mM AsA, 0.5 mM GSH in presence or absence of selenate stress. Averages of cell growth from three independent experiments ($n = 3$) are shown. Error bar represents SE. Bars with the same letters are not significantly different at $P < 0.05$.

and supplementation of proline also increased APX activity under Cd stress. It has been reported that the APX, CAT, and POX activities are increased in response to salt stress and supplementation of proline

also increases APX, CAT, and POX activities under salt stress on rice cultivars and suggest that proline detoxifies H₂O₂ by enhancing APX, CAT, and POX activities under salt stress [59,60]. In this study,

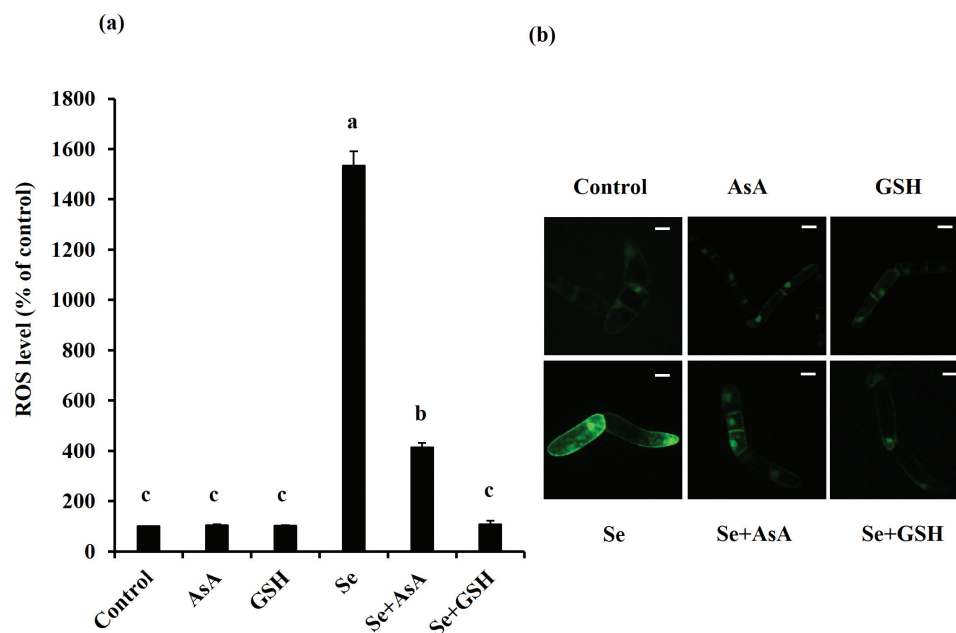


Figure 7. Effects of ascorbic acid (AsA) and glutathione (GSH) on intracellular ROS accumulation in BY-2 cells under selenate stress. (a) Intracellular ROS levels in 4-day-old Se-unadapted tobacco BY-2 suspension cells under selenate stress in the presence or absence of AsA and GSH. (b) Representative fluorescence images of 4-day-old Se-unadapted tobacco BY-2 suspension cells under selenate stress in the presence or absence of AsA and GSH. Scale bar = 20 μ m. The percentage of fluorescence intensity is shown as a ROS level. Averages from three independent experiments ($n = 3$) per bar are shown. Bars with the same letters are not significantly different at $P < 0.05$.

selenate stress increased activities of APX, CAT, SOD, and POX (Figure 4). Exogenous application of proline increased the APX and POX activities (Figure 4(a,d)) but did not affect either ROS accumulation (Figure 3(a)) or the lipid peroxidation (Figure 3(c)) in the BY-2 cells under selenate stress. In this study, proline enhanced APX and POX activities (Figure 4(a,d)) and also accelerated selenate accumulation (Figure 5). Taken together with the result that proline did not mitigate selenate stress (Figures 1 and 2), these results suggest that the increment of APX and POX activities by exogenous proline is not sufficient to suppress selenate stress due to selenate accumulation.

Both AsA and GSH are known as a vital component for the detoxification of ROS in plants [61–63]. Exogenous application of AsA improves phytotoxic effects of selenium on rice by reducing oxidative damage [64]. Jung et al. [65] reported that exogenous application of GSH alleviates heavy metal-induced oxidative damage and enhances heavy metal stress resistance in rice plants. In this study, the exogenous application of AsA and GSH significantly decreased the ROS accumulation in the selenate-stressed cells (Figure 7). However, GSH application significantly mitigated the growth inhibition by selenate (Figure 6) but AsA application did not significantly alleviate the growth inhibition (Figure 6). It has been reported that interference of GSH synthesis in plants by selenate or other Se compounds may diminish plant defense against hydroxyl radicals and oxidative stress [9]. Hence, in this study, the exogenous application

of GSH might increase the GSH level in BY-2 cells and the increased GSH might suppress the selenate toxicity. These results suggest that the application of proline does not always mitigate growth inhibition by heavy metals and suggest that the growth inhibition by selenate is not accounted for by ROS accumulation.

Conclusion

The presented results indicate that selenate induces cell death and inhibits growth in BY-2 cells while selenate elevates antioxidant enzyme activities and that proline enhances antioxidant enzymes such as APX and POX and also selenate accumulation. It is concluded that proline improves antioxidant activities and enhances selenate uptake and that the balance may account for growth inhibition and cell death.

Author's contribution

D. M., S. M., and Y. M. planned the experiments; M. K. performed the experiments; D. M., M. S. R., and E. O., assisted in experiments; T. N., Y. N., and S. M. contributed to discussions; M. K., S. M., and Y. M. wrote the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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