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# Selenium invoked antioxidant defense system in *Azolla caroliniana* plant

## Sistema de defensa antioxidante inducido por selenio en *Azolla caroliniana*

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**Abstract.** Plants of *Azolla caroliniana* were treated with different selenium concentrations (0, 1, 2, 5, 7, 10 ppm) for seven days. Selenium (Se) content in *Azolla* plants increased significantly with increasing Se concentrations in the culture media up to 5 ppm. This indicated that *Azolla* plants were a good accumulator for Se. Selenium accumulation determined changes in *Azolla* biomass, doubling time and relative growth rates. Treatment of *Azolla* plants with low concentrations of Se (1 ppm) resulted in a significant increase in biomass. This was accompanied by a reduction in hydrogen peroxide and malondialdehyde (MDA) contents; the decrease percentages were 78% and 60%, respectively at 1 ppm Se in comparison with the control. At higher Se concentrations (>5 ppm), there was a significant increase in $H_2O_2$ and MDA contents, these increases were 3.2- and 2.8-fold at 10 ppm Se in comparison to controls, respectively. Compared to that in controls, total ascorbate as well as total glutathione contents, were significantly increased. The activity of the GR enzyme was significantly increased in *Azolla* plants with addition of different concentrations of Se. The increase was 2.2- and 3.4-fold at 2 and 7 ppm Se, respectively. The addition of high concentrations of Se (>5 ppm) to the growth media resulted in a significant increase in the GSH-PX and APX activities in *Azolla* plants. Thus, addition of Se affects Azolla plants, and these effects change from beneficial to toxic, as reflected in the metabolism and growth of the plants.

**Keywords:** Selenium; *Azolla caroliniana*; Ascorbate; Glutathione; Antioxidant enzymes; Glutathione peroxidase.

**Resumen.** Plantas de *Azolla caroliniana* fueron tratadas con diferentes concentraciones de selenio (0, 1, 2, 5, 7, 10 ppm) por siete días. El contenido de selenio (Se) en las plantas de *Azolla* se incrementó significativamente al incrementarse las concentraciones de Se en el medio de cultivo hasta 5 ppm. Esto indicó que las plantas de *Azolla* pudieron acumular bien el Se. La acumulación de Se determinó cambios en la biomasa, la duración del experimento por generación y las tasas relativas de crecimiento de *Azolla*. El tratamiento de las plantas de *Azolla* con bajas concentraciones de Se (1ppm) resultaron en un incremento significativo en la biomasa. Esto fue acompañado por una reducción en los contenidos de $H_2O_2$ y malondialdehido (MDA); los porcentajes de reducción fueron 78% y 60%, respectivamente, a 1 ppm de Se en comparación con el control. A mayores concentraciones de Se (> 5 ppm), hubo un incremento significativo en los contenidos de $H_2O_2$ y MDA. Estos incrementos fueron de 3.2 y 2.8 veces a 10 ppm Se en comparación a los controles, respectivamente. Comparado a los controles, los contenidos de ascórbico y glutatión totales se incrementaron significativamente. La actividad de la enzima GR se incrementó significativamente en las plantas de *Azolla* con el agregado de diferentes concentraciones de Se. El incremento fue de 2.2 y 3.4 veces a 2 y 7 ppm Se, respectivamente. El agregado de altas concentraciones de Se (> 5 ppm) al medio de crecimiento determinó un incremento significativo en las actividades de la GSH-PH y APX en las plantas de *Azolla*. De esta forma, el agregado de Se afecta a las plantas de *Azolla*, y estos efectos cambian de benéficos a tóxicos, como se reflejan en el metabolismo y crecimiento de las plantas.

**Palabras clave:** Selenio; *Azolla caroliniana*; Ascórbico; Glutatión; Enzimas Antioxidantes; Glutatión peroxidasa.

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# INTRODUCTION

Selenium (Se) is an essential mineral for growth, reproduction, and disease prevention in all animals and humans. It has not been classified as an essential element for plants, although its role has been considered to be beneficial in plants capable of accumulating large amounts of this element (Terry et al., 2000). There are evidences that Se can exert beneficial effects on plants at low concentrations (Hartikainen et al., 2000; Simojoki et al., 2003) but be toxic at high concentrations (Marschner, 1995). It was revealed that Se, applied at low concentrations, enhanced growth and antioxidative capacity of both mono- and dicotyledonous plants. The growth-promoting response to Se was demonstrated in lettuce and ryegrass (Hartikainen et al., 1997) and soybean (Djanaguiraman et al., 2005).

Uptake and accumulation of Se by plants is determined by its chemical form (selenate and selenite) and concentration; soil factors such as pH, salinity and $CaCO_3$ content; the identity and concentration of competing ions, and the ability of plants to absorb and metabolize selenium (Pendias, 2001). Actively growing tissues usually contain the largest amounts of Se (Kahakachchi et al., 2004). Plants usually accumulate more Se in shoot than root tissues (Zayed et al., 1998).

In higher plants, metabolism of Se is closely related to that of sulphur due to their chemical similarity. Brown & Shrift (1982) reported that the non-specific incorporation of the selenoamino acids (selenomethionine and selenocysteine) into proteins is thought to be the major cause of Se toxicity in non-accumulator plants supplied with a high Se dose. Selenomethionine (SeMet) can be converted to volatile dimethylselenide (DMSe), offering a release valve for excess Se from the plant (Lewis et al., 1974). Meanwhile, selenocysteine (SeCys) can be converted in plants to elemental Se and alanine (Pilon et al., 2003).

High concentrations of Se were shown to provoke oxidative stress such as increased lipid peroxidation in plants (Hartikainen et al., 2000). The addition of Se to stressed plants can influence the activities or levels of antioxidants, and thus regulate the ROS levels. In plants, glutathione peroxidase is a powerful scavenger of $H_2O_2$ and lipid peroxide. Glutathione GSH-PX is believed to be a key enzyme which can be widely and robustly activated by Se in various plants exposed to diverse environmental stresses (Feng et al., 2013). Hartikainen et al. (2000) reported a dramatic increase in the activity of GSH-PX and inhibited lipid peroxidation in both young and old ryegrass seedlings treated with different levels of Se. In contrast, the activity of superoxide dismutase (SOD) diminished in response to Se addition (Xue & Hartikainen, 2000).

*Azolla* is an aquatic fern native to Asia and Africa. There is a symbiotic relationship between *Azolla* and the nitrogen fixer, cyanobacteria algae *Anabaena azollae*. This algae invades certain cavities on the dorsal lobes of the leaves, with true roots on the ventral surface. It is used as a biofertilizer to crops because of its ability to fix $N_2$ at high rates and low cost.

The main objective of this study was to investigate the accumulation of Se in *Azolla caroliniana* and its effects on growth and antioxidant system (enzymatic and non-enzymatic).

# MATERIALS AND METHODS

**Plant material and growth condition.** *Azolla caroliniana* Wild (known as water velvet) was provided by Prof. Weam El-Aggan in 1982 from the Catholic University of Louvain, Belgium. It was identified by Prof. Peters G.A., Kettering laboratory Yellow Springs, Ohio 45387. Plants were acclimated in the greenhouse of the Faculty of Science, Alexandria, in 2500 $cm^3$ polyethylene vessels which were filled with a nitrogen free, modified Hoagland solution (2/5 concentration, pH 5.1). In this solution, $KNO_3$ and $Ca(NO_3)_2$ were replaced by KCl and $CaCl_2$, respectively. The molecular composition of the normal Hoagland-Arnon (1950) solution is illustrated in Table 1.

Table 1. Molecular composition of the Hoagland-Arnon solution (1950).
Tabla 1. Composición molecular de la solución Hoagland-Arnon (1950).

| Products | Mol. Wt. | Concentration (mg/L) | Molarity (mM/L) | Normality (meq/L) |
|---|---|---|---|---|
| $KNO_3$ | 101.11 | 505.5 | 5 | 5 |
| $Ca(NO_3)_2.4H_2O$ | 236.16 | 1180.8 | 5 | 10 |
| $MgSO_4.7H_2O$ | 246.49 | 493.0 | 2 | 4 |
| $KH_2PO_4$ | 136.09 | 136.1 | 1 | 1 |
| $MnSO_4.H_2O$ | 169.02 | 1.538 | $9.1x10^{-3}$ | $1.82x10^{-2}$ |
| $ZnSO_4.7H_2O$ | 287.55 | 0.220 | $7.6x10^{-4}$ | $1.52x10^{-3}$ |
| $CuSO_4.5H_2O$ | 249.68 | 0.079 | $3.1x10^{-4}$ | $6.2x10^{-4}$ |
| $MoO_3.H_2O$ | 161.96 | 0.017 | $1.0x10^{-4}$ | - |
| $H_3BO_3$ | 61.84 | 2.860 | $4.6x10^{-2}$ | - |
| Fe(EDTA)Na | 367.05 | 30 | $8.2x10^{-2}$ | 0.245 $Fe^{++}$<br>0.082 $Na^{+}$<br>0.654 EDTA |

About 5 g (fresh mass) of *Azolla* were inoculated in each vessel from the stock material to make a new subculture. The plants were released from epiphytic microorganisms by thorough washing with distilled water. Cultures were grown in a growth chamber under a 16-h photoperiod at 1200 μmol/m$^2$/s irradiance (cool white fluorescent tubes) and temperatures (light/dark) of 28-30/20-25 °C (Stock culture). Plants were surface sterilized with 0.2% Clorox before use (El-Aggan, 1982), and then thoroughly washed with distilled water.

**Selenium treatment and growth estimation.** 5 g of *Azolla* plants were transferred to 250 cm$^3$ vessels containing 2/5 modified Hoagland solution, and different concentrations of selenium as sodium selenite (0, 1, 2, 5, 7 and 10 ppm) for 7 days.

The number of generations and doubling time [doubling time (DT) = duration of the experiment per one generation] were determined from the fresh mass and duration of experiment employing the expression given by Peters et al. (1979): n (final mass) = n$^o$ × 2G, where G = number of generations, n$^o$ = initial mass of *Azolla* plants (mass of inoculum). Relative growth rate (RGR) was calculated by using the formula of Subudhi & Watanabe (1981): RGR [kg/kg/d] = 0.693/DT.

**Determination of selenium in plant material.** Five grams of finely powder dry *Azolla* plants were placed in a 250 mL beaker, and 10 cm$^3$ of 1:1 (v/v) mixture of concentrated sulphuric acid and nitric acid were added (Krishnaiah et al., 2003). This solution was heated, until the mixture was clear. This solution was filtered and concentrated to 5 cm$^3$, cooled and diluted up to 50 cm$^3$ with deionized water. Then the standard procedure was applied to 1 cm$^3$ of this solution. One cm$^3$ plant extract was transferred into 25 mL calibrated flasks, to which 5 mL of concentrated HCl and 2.0 cm$^3$ of 2, 4-dinitrophenyl hydrazine hydrochloride (2,4-DNPH)–*N* (1- naphthyl) ethylenediamine dihydrochloride (NEDA) reagent mixture were added. The mixture was allowed to stand for 10 min with occasional shaking for completion of the reaction. The contents were diluted with water, and the absorbance was measured at 520 nm against the corresponding reagent blank. The calibration graph was constructed.

**Estimation of hydrogen peroxide ($H_2O_2$).** Hydrogen peroxide content was determined according to Velikova et al. (2000). Plant tissues (50 mg) were homogenized in an ice bath with 5 cm$^3$ of 0.1% (w/v) trichoroacetic acid (TCA). The homogenate was centrifuged at 12000 rpm for 15 min. The supernatant was used in the assay for $H_2O_2$. The reaction mixture contained 0.5 cm$^3$ plant extract, 0.5 cm$^3$ of 10 mM potassium phosphate buffer (pH 7) and 0.1 mL 1 M KI. The content of $H_2O_2$ was calculated by comparison with a standard calibration curve using different concentration of $H_2O_2$, and the results were expressed as **μ**mol $H_2O_2$ g/f.m.

**Estimation of lipid peroxidation.** Lipid peroxidation was measured as the amount of malondialdehyde (MDA) produced by the thiobarbituric acid (TBA) reaction, as described by Zhange et al. (2007). About 1.5 cm$^3$ extract was homogenized in 2.5 cm$^3$ of 5% TBA dissolved in 5% trichloroacetic acid (TCA). The mixture was heated at 95 °C for 15 min, and then quickly cooled on ice. After centrifugation at 5000 rpm for 10 min, the absorbance of the supernatant was measured at 532 nm. Correction of non-specific turbidity was made by subtracting the absorbance value measured at 600 nm. The concentration of MDA was calculated in term of μmol MDA g/f.m.

**Estimation of ascorbate and glutathione level.** A homogenate was prepared with 0.5 g of plant sample with 5ml of 5% TCA, which was then centrifugated at 10000 rpm for 10 min. The supernatant was used for the estimation of GSH and ascorbate. Reduced glutathione was determined using 5,5-dithiobis (2-nitrobenzoic acid) (DTNB, Sigma) as a reagent (Moron et al.,1979). Total glutathione was done by the method of Griffith (1980). The estimation of ascorbate was done by the method of Oser (1979) using sodium molybdate reagent.

**Preparation of enzyme extract and assay of enzyme activity.** Leaf tissues (0.5 g) were ground to a fine powder in liquid $N_2$, and then homogenized in 2 cm$^3$ of 50 mM potassium phosphate buffer (pH 7.0), 1.0 mM EDTA, 1.0 mM d-isoascorbic acid, 2% (w/v) polyvinylpyrrolidone (PVP) and 0.05% (w/v) Triton X-100 using a chilled pestle and mortar following Gossett et al. (1994). The homogenate was centrifuged at 10000 rpm for 10 min at 4 °C, and the supernatant was collected and used for the enzyme assay. Protein concentrations in the enzyme and plant extracts were determined by the method of Bradford (1976) using defatted BSA (Sigma, fraction V) as a standard.

**Ascorbate peroxidase (APX, EC 1.11.1.11)** was assayed as described by Nakano and Asada (1981). The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 0.2 mM EDTA, 0.5 mM ascorbic acid and 0.25 mM $H_2O_2$. The reaction was started at 25 °C by the addition of $H_2O_2$ after adding the enzyme extract. The decrease in absorbance at 290 nm for 1 min was recorded, and the amount of ascorbate oxidized was calculated from the extinction coefficient 2.8 mM$^{-1}$ cm$^{-1}$.

**Glutathione peroxidase (GSH-PX, EC 1.11.1.9).** The activity was modified from Flohe and Gunzler (1984). For the enzyme reaction, 0.2 cm$^3$ of the supernatant was mixed with 0.4 cm$^3$ GSH (Sigma product), and 2.2 cm$^3$ of 0.32 M $NaHPO_4$ and 0.32 cm$^3$ of 1.0 mM 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB, Sigma) were added for color development. The absorbance was measured with a spectrophotometer at 412 nm after 5 min. The enzyme activity was calculated as a decrease in GSH with reaction time as compared to that in the non-enzyme reaction.

**Glutathione reductase (GR, EC 1.6.4.2)** activity was determined at 25 °C by measuring the rate of NADPH oxidation as the decrease in absorbance at 340 nm (ℰ = 6.2 $mM^{-1}cm^{-1}$) following Halliwell and Foyer (1978). The reaction mixture (1 $cm^3$) consisted of 100 mM Tris-HCl (pH 7.8), 21 mM EDTA, 0.005 mM NADPH, 0.5 mM oxidized gtutathione (GSSG), and the enzyme. NADPH was added to start the reaction. For APX, GPX, and GR, the enzyme activity was expressed in terms of µmol/mg protein/min.

**Statistical analysis.** It was done using the Statistical Package for Social Sciences (SPSS/version 20) software. Data were analyzed using ANOVA. Whenever F tests were significant, mean comparisons were made using the Duncan test. The level of significance was 0.05.

# RESULTS

*Azolla* plants cultivated at various selenium concentrations (0, 1, 2, 5, 7, 10 ppm) showed different growth pattern. There was a significant increase in biomass of *Azolla* plants grown at 1 ppm Se. Thereafter, there was a steady decrease in biomass with increasing Se concentrations (≥5 ppm). At 1 ppm Se, the increase in *Azolla* biomass was about 30%, while at 7 ppm the decrease was about 68% compared to the control (Table 2). The doubling time (DT) and relative growth rate (RGR) were changed markedly with different concentrations of Se. The lowest DT appeared at 1 ppm Se (highest RGR), and the highest DT at 10 ppm Se (lowest RGR) on *Azolla* plants (Table 2).

*Azolla* plants accumulated Se efficiently during cultivation at different concentrations (Fig.1). Selenium content in *Azolla* increased significantly with increasing Se concentration in the culture media up to 5 ppm Se.

Table 2. Growth characterized by biomass, doubling time (DT), relative growth rate (RGR) and protein content of *Azolla* plants treated with different concentrations of Se (0, 1, 2, 5, 7, 10 ppm) for 7 days. Values are mean ± SD (n=3).
Tabla 2. Biomasa, duración del experimento durante una generación (DT), tasa relativa de crecimiento (RGR) y contenidos de proteínas de plantas de *Azolla* tratadas con diferentes concentraciones de Se (0, 1, 2, 5, 7, 10 ppm) por 7 días. Los valores son el promedio ± 1 SD de n=3.

| Treatments [ppm] | Biomass [g/culture] | DT [d] | RGR [kg/kg/d] | Protein content [mg/g d.m.] |
|---|---|---|---|---|
| **Control** | 10.34 a ± 0.84 | 6.8 a ± 0.55 | 0.1 a ± 0.019 | 30.27 a ± 2.46 |
| **1** | 13.38 b ± 1.09 | 5.2 a ± 0.42 | 0.13 a ± 0.026 | 50.56 b ± 4.10 |
| **2** | 10.75 a ± 0.87 | 6.5 a ± 0.53 | 0.11 a ± 0.021 | 30.94 a ± 2.51 |
| **5** | 5.00 c ± 0.41 | 14.0 b ± 1.14 | 0.05 b ± 0.009 | 20.71 c ± 1.68 |
| **7** | 3.30 c ± 0.27 | 21.2 c ± 1.72 | 0.03 b ± 0.006 | 10.80 d ± 0.88 |
| **10** | 1.11 d ± 0.09 | 63.1 d ± 5.12 | 0.01 b ± 0.002 | 6.32 d ± 0.51 |
| **P** | 0.001* | 0.0001* | 0.001* | 0.0001* |

Table 3. Changes in $H_2O_2$ content and lipid peroxidation (MDA) [µmol/g f.m.] in *Azolla* plants treated with different concentrations of Se (0, 1, 2, 5, 7, 10 ppm) for 7 days. Values are mean ± SD (n=3).
Tabla 3. Cambios en el contenido de $H_2O_2$, y peroxidación de lípidos (MDA) [µmol/g f.m.] en plantas de *Azolla* tratadas con diferentes concentraciones de Se (0, 1, 2, 5, 7, 10 ppm) por 7 días. Los valores son el promedio ± 1 SD de n=3.

| Treatments | Hydrogen Peroxide | MDA |
|---|---|---|
| **Control** | 0.36 a ± 0.04 | 3.40 a ± 0.41 |
| **1** | 0.08 b ± 0.01 | 1.35 b ± 0.16 |
| **2** | 0.23 a ± 0.03 | 1.46 b ± 0.18 |
| **5** | 0.32 a ± 0.04 | 3.79 a ± 0.46 |
| **7** | 0.81 c ± 0.10 | 5.55 c ± 0.67 |
| **10** | 1.16 d ± 0.14 | 9.56 d ± 1.15 |
| **p** | 0.001* | 0.001* |

Values followed by the same letter are not significantly different from control at P≥0.05.

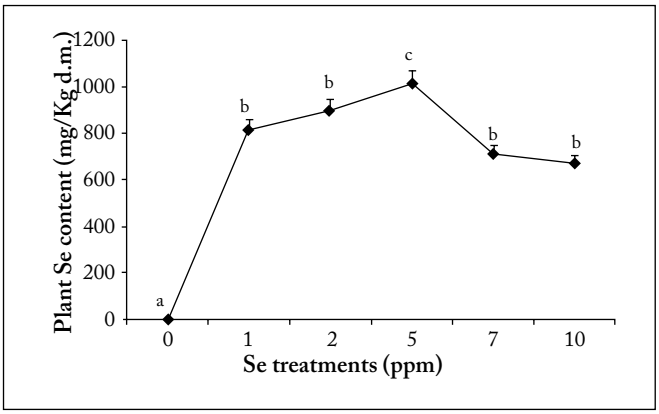


Fig. 1. Variation in total selenium content in *Azolla* plants treated with different concentrations of Se (0, 1, 2, 5, 7, 10 ppm) for 7 days. Values are mean ± SD (n=3).
Fig. 1. Variación en el contenido total de Se en plantas de *Azolla* tratadas con diferentes concentraciones de Se (0, 1, 2, 5, 7, 10 ppm) por 7 días. Los valores son el promedio ± 1 SD de n=3.

Table 4. Change in ascorbate, reduced glutathione, oxidized glutathione contents [nmol/g f.m.] and GSH/TG ratio in *Azolla* plants treated with different concentrations of Se (0, 1, 2, 5, 7, 10 ppm) for 7 days. Values are mean ± SD (n=3).
Tabla 4. Cambios en los contenidos de ácido ascórbico, glutatión reducido, glutatión oxidado [nmol/g f.m.] y relación GSH/TG en plantas de *Azolla* tratadas con diferentes concentraciones de Se (0, 1, 2, 5, 7, 10 ppm) por 7 días. Los valores son el promedio ± 1 SD de n=3.

| Treatments | Ascorbate | TG | GSSG | GSH | GSH/TG |
|---|---|---|---|---|---|
| **Control** | 3.39 a ± 0.41 | 10.65 a ± 0.94 | 0.95 a ± 0.15 | 9.70 a ± 1.04 | 0.91 a ± 0.14 |
| **1** | 4.22 a ± 0.51 | 17.72 b ± 1.56 | 4.68 b ± 0.74 | 13.04 b ± 1.40 | 0.74 b ± 0.12 |
| **2** | 6.59 b ± 0.79 | 19.15 b ± 1.69 | 8.74c ± 1.38 | 10.41 a ± 1.12 | 0.54 c ± 0.09 |
| **5** | 6.49 b ± 0.78 | 19.67 b ± 1.74 | 9.20 c ± 1.45 | 10.47 a ± 1.12 | 0.53 c ± 0.08 |
| **7** | 7.11 b ± 0.85 | 18.97 b ± 1.68 | 9.79 c ± 1.55 | 9.18 a ± 0.98 | 0.48 c ± 0.08 |
| **10** | 5.67ab ± 0.68 | 19.29 b ± 1.70 | 15.84 d ± 1.59 | 3.45c ± 0.99 | 0.18 d ± 0.07 |
| **P** | 0.013* | 0.001* | 0.001* | 0.021* | 0.018* |

Values followed by the same letter are not significantly different from control at P≥0.05.

Table 5. Changes in GSH-PX, APX and GR specific activity (µmol/mg protein/min) in *Azolla* plants treated with different concentrations of Se (0, 1, 2, 5, 7, 10 ppm) for 7 days. Values are mean ± SD (n=3).
Tabla 5. Variación en la actividad específica de GSH-PX, APX y GR (µmol/mg proteína/min) en plantas de *Azolla* tratadas con diferentes concentraciones de Se (0, 1, 2, 5, 7, 10 ppm) por 7 días. Los valores son el promedio ± 1 SD de n=3.

| Treatments | GSH-PX | APX | GR |
|---|---|---|---|
| **Control** | 7.66 a ± 0.92 | 8.68 a ± 0.77 | 2.07 a ± 0.22 |
| **1** | 9.42 b ± 1.13 | 8.27 a ± 0.73 | 3.04 a ± 0.33 |
| **2** | 9.93 b ± 1.19 | 7.66 a ± 0.68 | 4.64 b ± 0.50 |
| **5** | 11.39 c ± 1.37 | 13.32 b ±1.18 | 5.90 b ± 0.63 |
| **7** | 13.26 c ± 1.59 | 15.27 b ± 1.35 | 7.02 c ± 0.75 |
| **10** | 12.92 c ± 1.55 | 24.84 c ± 2.19 | 8.43 c ± 0.90 |
| **P** | 0.0156* | 0.001* | 0.012* |

Values followed by the same letter are not significantly different from control at P≥0.05.

In the present study, Se enhanced the total protein content in *Azolla* plants at the lowest concentration; the increase percent was about 67% at 1 ppm. However, higher concentrations of Se inhibited total protein contents (Table 2). This reflects the either stimulation or inhibition of *Azolla* biomass, DT and RGR at the different Se concentrations.

Addition of Se to the *Azolla*-growth medium resulted in a significant reduction in the generation of ROS. This determined a decrease in the $H_2O_2$ content at lower Se concentrations (≤2 ppm); the percentage of decrease was 78% at 1 ppm Se in comparison with the control. This was also accompanied by a decrease in malondialdehyde (MDA) content that is the decomposition product of lipid peroxidation. The percentage of MDA reduction was 60% at 1 ppm Se in comparison to the control. At higher Se concentrations (>5 ppm), there was a significant increase in $H_2O_2$ and MDA contents, the increases were of 3.2- and 2.8-fold at 10 ppm Se, respectively, in comparison to the control (Table 3).

In this study, the addition of Se to the *Azolla* growth medium can influence the activities or levels of antioxidants, and thus regulate the ROS levels. Total ascorbate and total glutathione as well as GSH contents were significantly increased in comparison to the control. The increase in total ascorbate and glutathione was 67% and 81%, respectively, at 10 ppm (Table 4). These increases in total glutathione and GSH contents were accompanied with a significant decrease in the glutathione redox potential (GSH /TG) at different Se concentrations in *Azolla* plants. This indicated an increase in the GR activity. The activity of the GR enzyme was significantly increased in *Azolla* plants with addition of different concentrations of Se (Table 5). The increase was 2.2- and 3.4-fold at 2 and 7 ppm Se, respectively.

There was a significant increase in GSH-PX and APX activities in *Azolla* plants by the addition of Se to the growth media at higher concentrations (≥5 ppm). The increase percentages were 73% and 76%, respectively, at 7 ppm compared to the control (Table 5). At lower Se concentrations, the change in APX activity was insignificant.

# DISCUSSION

In this investigation Se exerted a dual effect on growth of *Azolla* plants. At low concentrations, it stimulated the growth of plants, whereas at high dosages, it acted as a pro-oxidant and caused damage to the plant. Similarly, there is evidence that trace amount of Se can enhance the growth of some plant species (Chen et al., 2005; Djanaguiraman et al., 2005; Kumar et al., 2012). At high concentrations, Se acts as pro-oxidant and leads to drastic reduction in biomass (Hartikainen et al., 2000; Chen et al., 2008).

Selenium content in *Azolla* increased significantly with increasing Se concentrations in the culture media up to 5 ppm, indicating that *Azolla* was a good accumulator for Se. Selenium accumulation reflected by changes in *Azolla* biomass. The increase in biomass was obtained in plants exposed to low Se concentrations (≤2 ppm). At higher concentrations

of Se (≥7 ppm) in the culture media, Se accumulation decreased and this coincided with a reduction in *Azolla* biomass. The reduction in Se contents in *Azolla* plant at higher concentrations may be related to inhibition of Se uptake. This might be the result of a reduction in Se assimilation which required a H-donor increasing the competition between Se and $N_2$ on the hydrogen donor. It might also be related to volatilization of Se from the *Azolla* surface area. Ornes et al. (1991) reported that *Azolla caroliniana* absorbed Se quickly, and attained maximum tissue concentrations (1000 μg/g) after exposure to selenate at 5 ppm in comparison to other floating aquatic plants. Pilon-smits et al. (1999) recorded that *Hippuris vulgaris* L. and *Azolla caroliniana* willd were responsible for the two highest rates of both selenate and selenite volatilization.

Se enhanced the total protein content in *Azolla* plants at the lowest concentration. While higher concentrations of Se inhibited it, and this reflected the stimulation or inhibition of *Azolla* biomass, DT and RGR at the different Se concentrations. It was reported that Se accumulation enhanced the production of biomass, photosynthetic pigments and protein concentrations (Li et al., 2003; Chen et al., 2005, 2006 a, b). The higher Se concentrations (≥5 ppm) resulted in significant decreases in protein content of *Azolla* plants, which might be due to the toxic effects of Se stress (Chen et al., 2008).

It has been reported that the increase in Se concentration led to higher Se accumulation, and induced lipid peroxidation coupled with potassium leakage and decreases in biomass and photosynthetic pigments (Chen et al., 2008). The reduction of *Azolla* biomass and protein contents might be related to the generation of reactive oxygen species (ROS), and therefore to the destruction of plasma membranes and proteins. Severi (2001) found that both Na selenite and Na selenate generally decreased the growth and multiplication rate of *Lemna minor* at high concentrations; however, low concentrations actually increased the multiplication rate. It has been shown that Se can reduce lipid peroxidation at lower doses (Cartes et al., 2005; Djanaguiraman et al., 2005), while act as a pro-oxidant at higher dosages causing damage to plants.

In this study, the optimal dosages of Se (concentrations ≤2 ppm) reduced the levels of $H_2O_2$ and MDA either directly or indirectly via the regulation of antioxidants, especially of $H_2O_2$-quenchers (GSH-PX). The higher Se dosages increased production of ROS, which may be partially related to an imbalance in the levels of antioxidants. Feng et al. (2013) proposed that the increased ROS production at high Se levels may be related to an imbalance in the levels of GSH, thiols (-SH), ferredoxins (Fd.red) and/or NADPH, which can play vital roles in the assimilation of Se. Hartikainen et al. (2000) reported that low concentrations of Se inhibited lipid peroxidation in *Lolium perenne*, and this decrease coincided with an enhancement of growth. At high concentrations, Se acts as a pro-oxidant and leads to drastic reductions in yield. This dual effect of Se coincided with a promotion and inhibition of growth of *Azolla* plants indicating that Se may have particular biological functions through alteration of antioxidant defense systems.

Selenium can mimic sulphur, forming Se analogues of S compounds, replacing S in amino acids (methionine and cysteine) which leads to an incorrect folding of proteins and consequently to non-functional proteins and enzymes (Germ & Stibilj, 2007). This can help explain the toxicity of Se at higher concentrations in *Azolla* plants.

The significant increases in total ascorbate and total glutathione as well as GSH contents in Se-treated *Azolla* might be due to the high activity of GR which allowed the reducing agent (GSH) to eliminate ROS via GSH-PX or APX. Reduced GSH plays an important role in the antioxidant defence system of *Azolla*. This is because it not only participates in the regeneration of ascorbate via dehydroascorbate reductase but also can react with singlet oxygen and $OH^-$ radicals protecting protein thiol groups (Asada, 1994).

Mittler (2002) reported that a high reducing power of ASA and GSH is essential to quench excess ROS in plants. Thus, high levels of GR, monodehydro-ascorbate reductase (MDAR), dehydroascorbate reductase (DHAR) and NADPH are required. The enhancement of ascorbate and glutathione levels by Se in *Azolla* plants at different concentrations compared to controls reflects the enhancement of GSH contents to synthesize organic Se. This conclusion is supported by the results of Hasanuzzaman & Fujita (2011) who found that Se increased levels of ASA and significantly enhanced APX, DHAR, MDAR, GR and GSH-PX activities in rapeseed seedlings.

In this study, the significant decrease in glutathione redox potential (GSH/TG) with different Se concentrations in *Azolla* plants accompanied with a significant increase in TG and GSH indicated an increase in the GR activity. The reduction at the redox state of the glutathione pool was recorded by Molina et al. (2002) in heat-stressed plants.

The enhancement of GR activity allowed the reducing agent (GSH) to eliminate ROS via APX and GSH-PX. Increases in the activation/levels of APX, GSH, CAT, GR were also observed in the Se accumulator *Pteris vittala* after addition of excess Se (Feng & Wei, 2012).

GSH-PX is a powerful scavenger of $H_2O_2$ and lipid peroxide, with help of GSH; it is believed to be a key enzyme that can be widely and robustly activated by Se in various plants (Feng et al., 2013). The inhibition of $H_2O_2$ and lipid peroxidation coincides with the enhancement of growth in *Azolla* plants at low Se concentrations (≤2 ppm), which might be related to the increased activity of GSH-PX. At higher Se concentrations (>5 ppm) both GSH-PX and APX activities were significantly increased as well as $H_2O_2$ and lipid peroxidation compared to the control. These results indicate that the increase in the activities of antioxidant enzymes (GSH-PX and APX) was not sufficient to protect *Azolla* plants from Se-induced toxic damage. Similar results were obtained by Chen

et al. (2008). Takeda et al. (1997) reported that in absence of Se, the external $H_2O_2$ in *Chlamydomonas* cells is quenched by the APX and CAT enzymes. However, in the presence of Se, $H_2O_2$ is primarily scavenged by GSH-PX instead of APX and CAT. Also, Hartikainen et al. (2000) reported a dramatic increase in the activity of GSH-PX in both younger and older ryegrass seedlings treated with different levels of Se. Mitochondrial GSH-PX increased three-fold when fenugreek (*Trigonella Foenum-graecum* L.) plants were supplemented with Se (Sreekala et al.,1999). The positive relationship between Se concentration and GSH-PX activity suggests the presence of Se-dependent GSH-PX in plants (Hartikainen et al., 2000), and the increase in the activity of the GSH-PX enzyme in response to Se suggests a unique role for this enzyme in counteracting oxidative stress in plants.

In conclusion, the effects of Se on *Azolla* plants changed from beneficial to toxic, as reflected in the metabolism and growth of the plant. Although Se is harmful (toxic) for *Azolla* plants at high concentrations (≥5 ppm) leading to reductions in biomass, it can exert beneficial effects at 1 ppm by promoting plant growth.

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