RESEARCH ARTICLE

Selenium Linked Arsenic Tolerance Entails Induction of Phytochelatins, Amino Acids and Promotes Reduction of Arsenic Phytotoxicity and Uptake in Rice

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Abstract

Exposure of population through consumption of arsenic (As) tainted rice is a major problem over the world and has become an alarming situation in reference to the quantity and quality of agricultural produces together with human health. Selenium (Se) is a potent antagonist of As. Selenate (SeVI) supplementation markedly ($p<0.05$) reduced level of As and oxidative stress in roots and shoots as well as induced growth suggesting its beneficial role against As. Different species of phytochelatins (PC_2, PC_3 and PC_4) were analyzed during Se(VI) and As(V) interaction. Total PCs in roots were in the order of control<Se<As<As+Se, suggesting thiol's role in As tolerance and detoxification during As/Se interaction. The phytochelatin synthase (PCS) activity respond according to PCs contents along with cysteine synthase (CS), serine acetyltransferase (SAT), γ-glutamyl cysteine synthase (γ-ECS) and γ-glutathione transferase (γ-GT) exhibiting their maximum activities at Se(VI) added As(V) stressed plants. Supplementation of Se(VI) during As(V) stress significantly ($p<0.05$) reduced O_2•−by 20 and 21%, H_2O_2 by 25 and 16%, MDA by 44 and 33%, NO level by14 and 12% and NOX activity by 13 and 19%, thiolic enzymes such as SAT by 25 and 13%, CS by 24 and 28%, γ-ECS by 7 and 11% and γ-GT by 19 and 5%, in roots and shoots, respectively in comparison to As alone treated plants. Arsenic drastically reduced the amount of essential amino acids (EAAs), however, Se(VI) improved their (isoleucine, lysine, leucine, threonine and phenylalanine) level during As(V) stress. Some stress responsive non-essential amino acids (proline, cysteine, glutamic acid and glycine) increased during Se(VI) and As(V) exposure and few amino acids (arginine, serine and aspartic acid) recovered to the level of control implying the role of Se(VI) mediated remediation of oxidative stress, which probably leading to lower peroxidation of amino acids due to lesser free radicals. Thus, Se(VI) imparts As(V) tolerance through the modulation of amino acids and phyochelatins in rice plant.

Keywords: Arsenate, Amino Acids, Antioxidants, Phytochelatins, ROS staining, Selenate.

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Introduction

Arsenic (As) is a ubiquitous and highly toxic contaminant to all life forms and also recognized as a non-threshold human carcinogen. Groundwater As contamination is severe in South and South-East Asia due to its natural geogenic origin (Meharg 2004). Over a decade, the arsenic problem is highly considered a major environmental challenge (Tripathi *et al.* 2012). Rice is a substantial food for half of the world's population and irrigation of rice crop with as contaminated water leads to food chain contamination (Moore *et al.* 2010). In the terrestrial environment, inorganic species of As [arsenate (AsV) and arsenite (AsIII)] are mostly present in the environment and taken up by the plants with the help of their selective transporters (Tripathi *et al.* 2012). Arsenate (AsV) is transported through high affinity phosphate transporter, whereas arsenite (AsIII) go through the aquaporins transporter i.e. Nodulin 26-like intrinsic proteins (NIPs) in the rice (Awasthi *et al.* 2017; Lindsay and Maathuis, 2017).

Intra-conversion of As from one form to another form the plants causes oxidative stress by production of reactive oxygen species (ROS), leading to growth reduction, damage and biomolecule degradation (Chauhan *et al.* 2017). A number of arsenomics analyses have been done earlier using metabolomic, genomic and proteomic approaches in different plants species during As stress, which implied the importance of thiol metabolism and antioxidant defense system for As tolerance and detoxification in plants (Awasthi *et al.* 2021; 2022). Several stress-responsive amino acids are also known to buildup in plants on As exposure (Dwivedi *et al.* 2010, Tripathi *et al.* 2013a). Several amino acids like cysteine, glutamic acid and glycine are involved in glutathione (GSH) and phyochelatin (PCs) bio-synthesis, which are required for As complexation within the plant (Tripathi *et al.* 2013a, b).

Selenium (Se) is an essential nutrient element for humans and is considered beneficial for plants as well. Selenium is a cofactor of glutathione peroxidase (GPX) enzyme to ameliorate heavy metal induced phytotoxicity in plants. Selenocysteins are the major constituent of selenoenzymes or selenoproteins in organisms those require Se (Chauhan *et al.* 2022). Selanate [Se(VI)] and selenite [Se(IV)] are the predominant forms of the Se

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in the terrestrial environment. Selanate is transported through sulphate transporter, while silicon influx transporter (OsNIP2;1) is known for Se(IV) uptake in rice (Zhao *et al.* 2010). Selenium is concerned with detoxification of heavy metals by ameliorating oxidative stress and reducing metal uptake (Chauhan *et al.* 2017, Kumar *et al.* 2013). Besides, several studies have been performed to examine the effect of Se(IV) on As(III) uptake and associated phytotoxicity in rice (Chauhan *et al.* 2017), but Se(VI) and As(V) interaction is yet to be explored.

The asian rice is not only enriched in Se, but also retain high As in As contaminated areas (Meharg 2004, Tuli *et al.* 2010). The present study deals with the interactive effect of Se(VI) and As(V) on ROS measurement, amino acids response, thiolic ligands, including phytochelatins (PCs) and related enzymes in the rice. It was implicit that Se supplementation may outcome in improved growth, As reduction and amelioration of As induced oxidative stress through sustaining the balance of amino acids, thiols, including PCs to attain efficient As tolerance and detoxification in rice. Consequently, the present study was premeditated to inspect the Se(VI) mediated As(V) detoxification/tolerance in rice plants by evaluating the response of plant growth, accumulation of As and its associated toxicity, amino acids and amino acids and phytochelatins.

Material and Methods

Plant material and experimental conditions

Oryza sativa L. var. Triguna were collected from Chinsurah, Rice Research Station, West Bengal. The collected seeds were disinfected with 0.1% $HgCl_2$ solution for 30 s followed by washing with milli-Q water for 5–6 times. Germinated seedlings were transferred into Hewitt nutrient medium having pH 5.5 and left for acclimatization for 7 d under controlled conditions of temperature, relative humidity, light intensity (Liu *et al.*, 2004). After acclimatization, plantlets were exposed to different treatments *viz.,* control (no As), Selenate (SeVI), Arsenate (AsV), and AsV+SeVI have been given to the plant for 15 d. Two times in a week, nutrient solution were changed. The experiment was performed in triplicate (each replicate contained 10 rice seedlings). After completion of 15 d the harvesting of plant were carried out. Plants were washed with milli-Q, gently blotted and for further experiments, stored at -80^{0}C after frozen in liquid nitrogen.

Arsenic and selenium quantification and quality control

For estimation of total As, dried plant samples (1 g) were powdered and digested in 3 mL HNO_3 at 120 °C for 6 h (Dwivedi *et al.* 2010). The level of As was quantified by an inductively coupled plasma mass spectrometer (Agilent 7500cx). The standard reference material of As (Agilent, Part # 8500–6940) was used for each analytical batch's calibration and quality assurance. Rice flour NIST 1568a was used as a reference material with known spiked samples, and recovery of total As was 85.3 % (±2.8; n=5) and 89.5 % (±3.1; n=5), respectively. The detection limit of As and Se was 1 µg L^{-1}. Rhodium (Rh) and stannous (Sn) were used as internal calibration standards.

Estimation of superoxide radical, hydrogen peroxide, lipid peroxidation, ion leakage, nitric oxide and NADPH oxidase

The rate of superoxide radical ($O_2^{\bullet-}$) production was measured following the method of Chaitanya and Naithani (1994) by its capacity to reduce nitro blue tetrazolium (NBT) and is expressed as ΔA540 min^{-1} mg^{-1} protein. For estimation of hydrogen peroxide (H_2O_2) levels, plants were homogenized in ice cold 50mM potassium phosphate buffer (pH 7.0) containing 0.1% Triton X-100 and 1% PVP (w/v) (Milosevic and Slusarenko 1996) and the level of H_2O_2 was measured according to Pick (1986). The assay was based on horseradish peroxidase-dependent oxidation of phenol red by H_2O_2 leading to the formation of a compound at alkaline pH, which exhibited significant absorbance at 600 nm. Lipid peroxidation was determined by estimation of the malondialdehyde (MDA) content following the method of Heath and Packer (1968). The amount of MDA was calculated by the difference in absorbance at 532 and 600 nm using the ε of 155mM^{-1} cm^{-1}. The ion leakage was measured in terms of electrical conductivity (EC) according to Devi and Prasad (1998). Metalloid exposed plants were washed with deionized water (Millipore, USA) and 500 mg of plant material was then transferred to 100 mL of deionized water for 24 h to facilitate maximum ion leakage and the EC of the water was recorded. The level of nitric oxide (NO) was measured by the determination of nitrite (NO_2•–) concentration using Griess reagent. Samples (0.2) were incubated with 1.8 mL of 100 mM potassium phosphate buffer (pH 7.0) and 0.2 mL of Griess reagent (1% sulfanilamide and 0.1% N-1-naphthylethylenediamine dihydrochloride in 5% phosphoric acid) at room temperature for 10 minutes. Absorbance of the reaction mixture was recorded at 540 nm and the concentration of NO was determined using a standard curve prepared using the known concentrations of sodium nitrite (Green *et al.* 1982).

For the assay of NADPH oxidase (NOX; EC 1.6.3.1), control and metalloid–exposed plants were homogenized in 20 mM HEPES (pH 7.0) containing 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 2.5% polyvinylpyrrolidone (PVP) and 5% glycerol under chilled conditions. Homogenate was centrifuged at 12,000 x g for 15 minutes at 4°C. The protein content of the supernatant was measured following Bradford (1976). NADPH-dependent O_2•– generation by the enzyme NOX was measured using NBT as an electron acceptor (Bielski *et al.* 1980) whose reduction was monitored at 530 nm. Monoformazan concentrations (and therefore O_2•– concentrations) were calculated using an extinction coefficient of 12.8 mM^{-1} cm^{-1}.

Histochemical detection of superoxide radicals and hydrogen peroxide

In vivo H_2O_2 accumulation was analyzed using 3,3'-diaminobenzidine (DAB) by following the protocol of Thordal-Christensen *et al.* (1997). The stained roots and shoots were observed with MZ 16 Leica stereomicroscope (4X) and NBT stained roots were photographed.

Phytochelatin analysis

In 0.2 g of plant tissue (root and shoot) was taken for analysis and the precolumn derivatization of thiol compounds with

monobromobimane (mBBr) was based on the methods as described by Minocha *et al.* (2008). The derivatized samples were filtered with 0.45 µm nylon syringe filters for HPLC analyses. In 5 µL of the filtered sample was used for analysis on a Waters Binary gradient HPLC system with accessories module 2475, (Waters Milford, MA, USA) with Atlantis® T3 5µm 4.6x100 mm column. Chromatograms were integrated using Empower 2 HPLC software v6.0. Thiol compounds were separated by using solvents (A) 99.9% ACN and (B) 89.9% water + 10% ACN, both containing 0.1% TFA by volume. Standard samples of cysteine, GSH and PCs (PC_2, PC_3 and PC_4; Sigma-Aldrich, USA) were run to identify the peaks. The Concentration of PCs was estimated in terms of GSH equivalents and is expressed as µmoles of GSH equivalents g^{-1} fw.

Assay of Phytochelatin Synthase

For analysis of phytochelatin synthase (PCS; EC 2.3.2.15) activity, 0.2 g of plant material was homogenized in 1-mL of chilled Tris buffer (300 mM; pH 8.0) and centrifuged at 10,000 × g for 10 minutes. The supernatant was used in reaction for PCS assay according to Tripathi *et al.* (2012). After reaction, mixture was cleared by centrifugation and the supernatant was assayed for PCs synthesis by HPLC following pre-column derivatization with mBBr as mentioned above. Enzyme activity is expressed as nmoles PC_2 min^{-1} mg^{-1} protein.

Enzymes of Thiol Metabolism

Control and metalloid exposed plants were homogenized in buffers specific for each enzyme under chilled conditions. Protein content of the supernatant was measured following the method of Bradford (1976). The assay of serine acetyltransferase (SAT, EC 2.3.1.30), cysteine synthase (CS; EC 2.5.1.47), γ-Glutamyl cysteine synthase (γ-ECS, EC 6.3.2.2) and γ-glutamyl transpeptidase (γ-GT; EC 2.3.2.2) was performed by following Blaszczyk *et al.* (2002), Saito *et al.* (1994), Seelig and Meister (1984) and Orlowski and Meister (1973) respectively.

Amino Acid Analysis

The pico tag method was followed for estimation of amino acids on the Waters-HPLC system (detailed methodology provided in supplementary material) by following Bidlingmeyer *et al.* (1984). In an oven, the homogenized rice plant sample (200 mg) were hydrolyzed in 6 N HCl for an hour at 150°C. Samples were then filtered for further analysis. 10 mL of samples and standard (1-mL of 2.5 mmole in 0.1 N HCl) were dried in a vacuum oven at 55°C for 30 minutes at 75 milli torr. This was redried twice by adding 20 mL of redrying solution (Ethanol: Triethylamine: Water in a ratio of 2:1:2). Then samples were derivatizated by adding derivatization reagent (20 mL) (Ethanol: Triethylamine: Water: Phenyoisothiocynate, 7:1:1:1) and again vacuum dried. These samples were diluted to 1-mL with pico tag sample diluent, filtered (0.22 mm syringe filters). The separation was carried out at 40°C using a Pico Tag amino acid C18 column. For each sample, 20 mL of extract was injected and the column was eluted at 1-mL min^{-1}, with an optimized gradient established using solvents A (0.14 M sodium acetate, containing 0.05% triethylamine and 6% acetonitrile, pH 6.40) and B (60% acetonitrile in water). A step-by step gradient was used with an increase of proportion of solvent B until it reached 46% during 10 minutes, followed by an increase up to 100% in 5 min, with a flux of 1-mL min^{-1}. The column was then cleared and optimized to 100% A for 8 minutes at 1 mL $minutes^{-1}$. The amino acids analyzed were methionine (Met), lysine (Lys), leucine (Leu), threonine (Thr), valine (Val), phenylalanine (Phe), isoleucine (Ile), arginine (Arg), tyrosine (Tyr), aspartic acid (Asp), serine (Ser), histidine (His), glutamic acid (Glu), proline (Pro), glycine (Gly), cysteine (Cys) and alanine (Ala). Chromatograms were integrated using Empower 2 HPLC software v6.0.

Results and Discussion

Effect of Se(VI) on growth parameter and metal uptake during As(V) stress

Arsenic threat to paddy crops has become a worldwide problem due to accumulation in rice grains and its harmful effects on growth, yield and nutritional quality (Lindsay and Maathuis 2017). Changes in growth parameters were measured in respect to length and biomass of the plant during As(V) and Se(VI) interaction in rice (Fig. S1 a,b). A remarkable decline in length (8 and 5%) and biomass (35 and 24%) was observed in 15 days As (50 µM) exposed rice root and shoot, respectively (Fig. S1). Arsenic treatment considerably ($p<0.05$) decreased the length and biomass of rice roots and shoots demonstrating its toxic nature, which is in accordance with the earlier findings on rice (Srivastava *et al.* 2018). Studies on As toxicity amelioration should be conducted in rice to consume the safe rice. Hence, Se(VI) assisted As(V) tolerance was studied in rice during the present study. The presence of Se(VI) (0.5-2.5µM) during As(V) stress caused better growth and improved biomass signifying either an antagonistic behavior of Se(VI) with As(V) or with their other species after bio-transformation. Se(VI) supply is advantageous for the growth of rice plant; 2.5 µM Se(VI) level during As(V) exposure was most favorable for the plant growth and biochemical analysis, further than that Se(VI) level turned down the growth parameter during As stress. The potential basis behind better rice growth at As+Se exposure might be due to lesser As uptake due to competition between Se and As uptake and As associated toxicity.

Various reports indicated that Se supply uphold growth of the plant by alleviating biotic and abiotic stresses and maintaining nutrient balance (Kaur *et al.* 2014). Besides, As(V) prominently affected the photosynthetic pigments. Se(VI) supply more significantly increased the total chl, chl a, chl b and carotenoid by 24, 28, 22 and 6%, respectively (Fig. S2) during As(V). Photosynthetic pigments were also altered in rice during As stress (Chauhan *et al.* 2020) Se(VI) also promotes the plant growth and photosynthetic pigments in mungbean (*P. aureus* Roxb.) like rice during As stress (Malik *et al.* 2012).

The highest accumulation of As was found in roots (981 mg kg^{-1} dw) followed by shoots (109 mg kg^{-1} dw) at 50 µM As (Table 1). Se(VI) addition exhibited markedly ($p<0.05$) reduced As level in roots and shoots, maximum being of 51% and 46% at 2.5 µM Se(VI) during As stress. Similar studies suggested that As(V) accumulation was decreased in rice root and shoot during Se supplementation (Chauhan *et al.* 2017). It seems that

Table 1: Effect of Se supplementation on uptake of As and Se concentration in root and shoot of rice plant under different As treatments. All the values are means of 3 replicate (n=3) ±S.D. ANOVA significant at p<0.01. Different letters within the same column indicate significantly different values between treatments (DMRT, p<0.05).

Treatments	As (mg kg^{-1} dw)		Se (mg kg^{-1} dw)	
	Root	Shoot	Root	Shoot
Control	-	-	-	-
50 µM As	980.67a ± 54.6	108.66a ± 7.56	-	-
0.5 µM Se	-	-	25.8a ± 2.8	11.2b ± 2.8
1 µM Se	-	-	46.25b ± 7.3	21.2c ± 2.5
2.5 µM Se	-	-	145.5d ± 14.7	59.07e ± 6.5
50 µMAs + 0.5 µM Se	826.6b ± 37.7	97.4b ± 9.82	23.8a ± 4.37	8.1a ± 1.3
50 µMAs + 1 µM Se	653.3c ± 49.8	79.3c ± 5.59	43.3b ± 6.7	14.3b ± 1.6
50 µMAs + 2.5 µM Se	476.18d ± 10.8	58.7d ± 4.3	113.6c ± 8.7	38.6d ± 5.7

antagonistic interaction between As(V) and Se(VI) would exists because these species are readily reduced to As(III) and Se(IV) in anaerobic condition, further reduced As and Se species are known to share the same transporter (silicon influx transporter) (Malik *et al.* 2012).

Effect of Se(VI) supplementation on As(V) induced oxidative stress

Oxidative stress indicating parameters ($O_2^{\bullet-}$, H_2O_2, MDA, EC, NO and NOX) were measured during As(V) and Se(VI) interaction in roots and shoots of rice (Fig. 1 a–f). All the parameters *viz.*, $O_2^{\bullet-}$, H_2O_2, MDA, EC, NO and NOX were raised in roots (7, 18, 137, 69, 42 and 16%) and shoots (2, 39, 65, 60, 19 and 90%), respectively during As(V) stress. The outcome of this study hence reveals that As induced oxidative stress apparently initiate the formation of ROS, NO and pro-oxidant enzyme NOX (Tipathi *et al.* 2012, Awasthi *et al.* 2018) causing the degradation of various biomolecules *viz.*, lipids, proteins and DNA (Patra *et al.* 2004). However, Se(VI) supplementation during As(V) stress significantly (p<0.05) decreased $O_2^{\bullet-}$ (20 and 21%), H_2O_2 (25 and 16%), MDA (44 and 33%), EC (46 and 34 %), NO level (14 and 12%) and NOX activity (13 and 19%) in roots and shoots, respectively in comparison to As alone treated plants. Studies indicated that Se overcome the oxidative stress by dismutation of $O_2^{\bullet-}$ to H_2O_2 during Al, Cd and Pb stress (Kushwaha *et al.* 2021, Pandey and Gupta 2018). Selenate addition ameliorates As(V) induced oxidative stress might be due to its role in lowering As uptake, ROS quenching and antioxidant enzymes regulation (Lima 2019, Chandra and Roychoudhury 2020).

H_2O_2 measurement in roots and shoots was also done by DAB staining (Fig. 2) and As treated roots acquired maximum staining for H_2O_2 detection followed by As+Se>control>Se exposed plants. The intensity of ROS staining was minimum for Se(VI) exposed plants. The differential root As accumulation may lead to varying degree of toxicity due to ROS production causing lipid peroxidation, cell death and damage (Tripathi *et al.* 2013). Selenium performs ROS quenching and control their production either directly or indirectly via regulation of antioxidants (Kaur *et al.* 2014). Various reports indicated that ROS level was highly reduced by ambient doses of Se, probably due to Se assisted activation of antioxidants, especially glutathione peroxidase (GSH-Px; H_2O_2 quenchers) (Feng *et al.* 2013, Chauhan *et al.* 2017). Selenium helps in reducing the levels of $O_2^{\bullet-}$ and H_2O_2 signifying a interruption in ROS reaction chain causing lesser damage to biomolecules of plant cell membranes thus restoring its integrity (Chauhan *et al.* 2019).

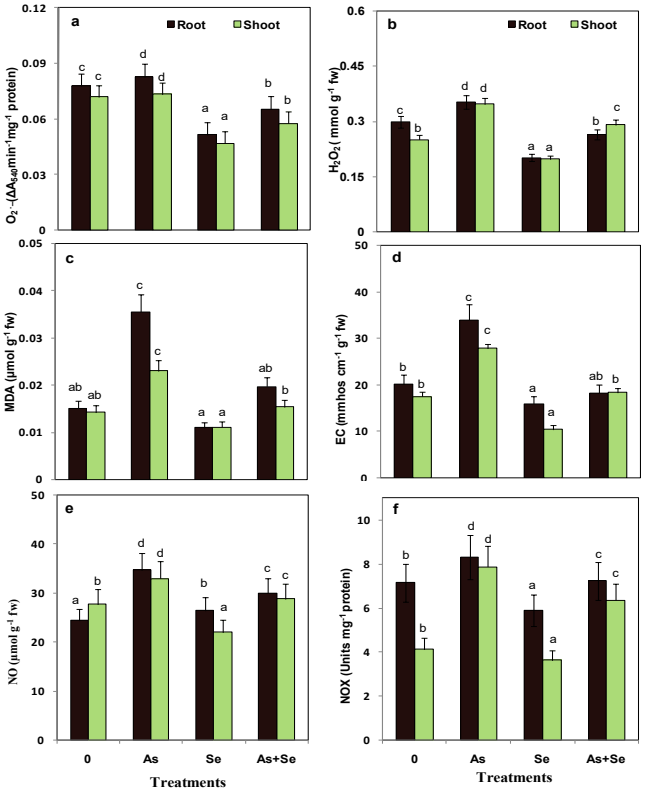

Fig. 1 (a-f): Changes in the level of superoxide radicals ($O_2^{\bullet-}$) (a), hydrogen peroxide (H_2O_2) (b), malondialdehyde (MDA) (c), ion leakage (EC) (d), nitric oxide (NO) (e), and NADPH Oxidase (NOX) (f) activities in roots and shoots of the rice seedlings after 15 d of treatments (0, 2.5 µM Se, 50 µM As and 50 µM As + 2.5 µM Se). All the values are means of 3 replicate (n=3) ± S.D. ANOVA significant at p ≤ 0.01. Different letters indicate significantly different values between treatments (DMRT, p≤0.05).

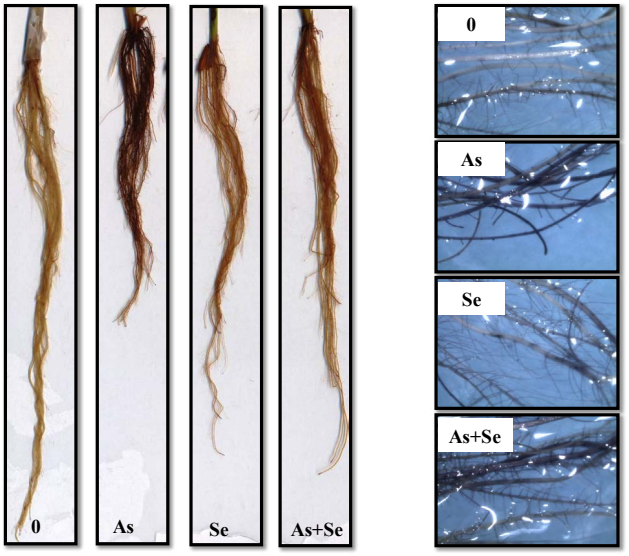

Fig. 2: Histochemical detection of hydrogen peroxide (H_2O_2) in roots and shoots of the rice seedlings stained with DAB after 15 d of treatments (0, 2.5 µM Se, 50 µM As and 50 µM As + 2.5 µM Se). The brown staining indicates the formation of polymerisation products when H_2O_2 reacts with DAB.

Effect of Se Supplementation on Phytochelatins and Phytochelatin Synthase During as Stress

The quantification of PCs in rice varieties has been recorded. The PCs chromatograms demonstrated the level of cysteine, GSH, PC_2, PC_3 and PC_4 along with some unidentified thiol peaks (Fig. 3 a-d'). Total PCs was increased by more than 19, 9 and 25 fold in root and about 3, 5 and 5 fold in shoot at As, Se and As+Se treatments, respectively than the control (Fig. S3). Synthesis of PC_2 showed induction of about 24, 10 and 28 fold in root (Fig. 3a-d) and percent induction of about 200, 244 and 265 in shoot at As(V), Se(VI) and As(V)+Se(VI) treatments, respectively than the control (Fig. 3a'-d'). Interestingly, during As(V) stress, the level of PC_3 and PC_4 was increased by more than 14 and 8 fold in roots and more than 5 and 2 fold in shoots than control. However, the level of PC_3 (3 and 5 fold) was lower than PC_4 (11 and 7 fold) both in root and shoot during Se(VI) stress than control. Besides, PC_3 and PC_4 levels were also maximum elevated during As(V)+Se(VI) interaction like PC_2, both in root (about 20 and 23 fold) and shoot (8 and 7 fold), respectively. The phytochelatins play central role in detoxification of As through complexation and different species of PCs (PC_2, PC_3 and PC_4) were noticed previously in rice during As stress (Tripathi *et al.* 2012, 2013). However, only total PCs were calculated during Se stress in rice (Kumar *et al.* 2013), while for the first time we have reported here the different species of PCs (PC_2, PC_3 and PC_4) during Se(VI) and As(V) interaction, which is of utmost important. Non-protein thiols (NPTs), possessing metal detoxifying and antioxidant potential contain glutathione (GSH) and PCs. During the present study, cysteine and NPTs increased during As and Se interaction (Fig. S3 a,b) like other studies (Malik *et al.* 2012, Tripathi *et al.* 2012) suggesting Se amendments improve the thiolic ligands against As toxicity. While GSH, GSSG and GSH/GSSG ratio decreased at metalloid exposure (Fig. S3c, d, e), suggesting the utilization of GSH in PCs formation (Tripathi *et al.* 2012, 2013).

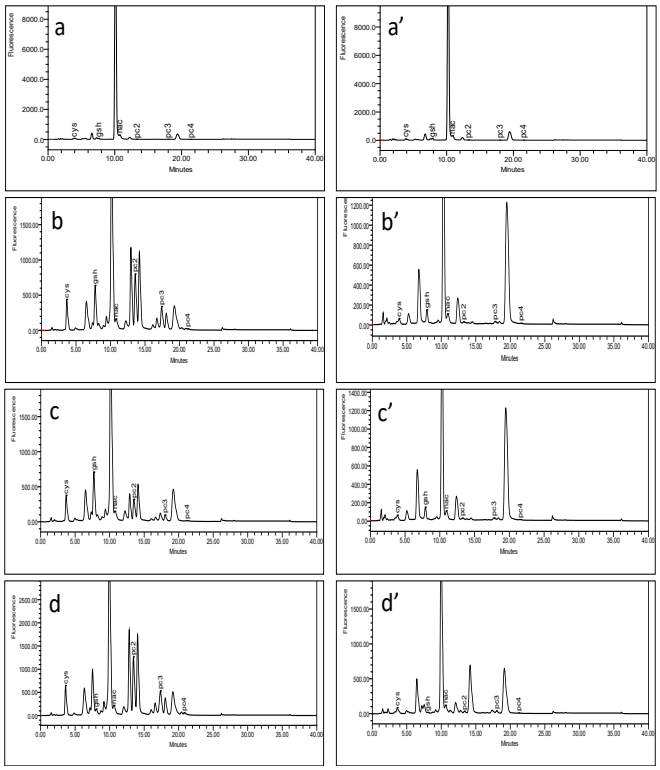

Fig. 3 (a-d): HPLC profiles of phytochelatins (PC_2, PC_3 and PC_4) in root (a-d) and shoot(a'-d') during control, As, Se and As+Se exposure.

Metalloids addition significantly modulates PCS activities in roots and shoots of rice seedlings. The rice plants respond according to PCs contents towards As(V), Se(VI) and As(V)+Se(VI) exposure by enhancing PCS activity in roots (114, 68 and 143%) and shoots (128, 114 and 175%, Fig. 4), respectively. The addition of Se(VI) to As(V) stressed plants showed maximum activities of PCS in roots and shoots. It is well established that activation of phytochelatin synthesis play important role in primary As detoxification strategies in plants (Srivastava *et al.* 2011, Tripathi *et al.* 2012, 2013). Induction of PCs was supported by a significant increase in PCS activity at different As(V) and Se(VI) exposure in rice (Kumar *et al.* 2013, Tripathi *et al.* 2012).

Effect of Se(Vi) Supplementation on Thiolic Enzymes During as(V) Stress

Se(VI) addition modulates thiolic enzymes *viz.*, SAT, CS, γ-ECS and γ-GT in roots and shoots of rice seedlings during As(V) stress. The enzymes of thiol metabolism impart positive response towards As(V) stress by inducing SAT (111 and 51%, Fig. 5a), CS (13 and 10%, Fig. 5b), γ-ECS (19 and 36%, Fig. 5c) and γ-GT (58 and 15%, Fig. 5d) in roots and shoots, respectively.

Many studies conferred that thiol metabolism plays a key role in As tolerance and detoxification. The enzymes of cysteine biosynthesis pathway *viz.*, SAT and CS correspond to each other in the present study, because cysteine is the central metabolite for As detoxification (Chauhan *et al.* 2017, Kumar *et al.* 2020).

During this study, Se(VI) addition showed increased activities of all these respective parameters in comparison to the control plants. Se(VI) addition to As(V) stressed plants also raised the

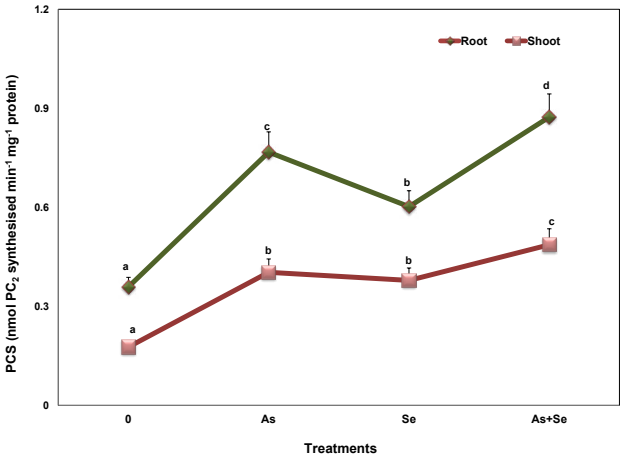

Fig. 4: Effect of As and Se interaction on the PCS activity in root and shoot of rice plant. All the values are means of triplicate ± S.D. ANOVA significant at $p \leq 0.01$.

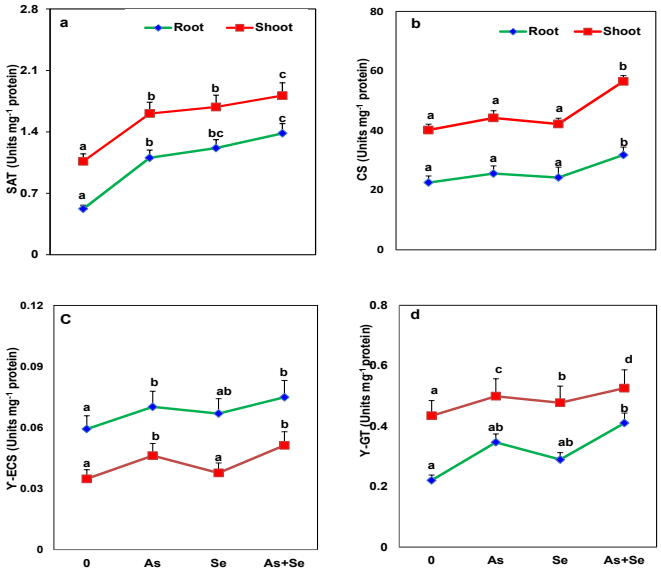

Fig. 5 (a-d): Changes in the activity of serine acetyl transferase (SAT) (a), cysteine synthase (CS) (b), γ-glutamylcysteine synthetase (γ-ECS) (c) and γ-glutathione transpeptidase (γ-GT) (d) in roots and shoots of the rice seedlings after 15 d of treatments (0, 2.5 µM Se, 50 µM As and 50 µM As+ 2.5 µM Se). All the values are means of 3 replicate (n=3)±S.D. ANOVA significant at $p \leq 0.01$. Different letters indicate significantly different values between treatments (DMRT, $p \leq 0.05$).

activities of thiolic enzymes *viz.*, SAT (25 and 13%, Fig. 5a), CS (24 and 28%, Fig. 5b), γ-ECS (7 and 11%, Fig. 5c) and γ-GT (19 and 5%, Fig. 5d) in roots and shoots, respectively than As(V) stressed plant. In support of this finding, various studies conclude that Se supplementation improved the thiols and related enzymes for the As tolerance (Kumar *et al.* 2016). Thus, it appears that Se alone or with As serves as antioxidants.

Effect of Se Supplementation on Amino Acids Profile During as Stress

The amino acid profiling was done in root and shoot of rice variety during As(V) and Se(VI) interaction (Fig. 6a, b). Most (Met, Leu, Lys, Thr, Val, Ile) of the essential amino acids (EAAs), were decreased both in As(V) treated root and shoot except Phe, however Se(VI) supplementation improved the EAAs either in the presence or absence of the As(V). In root, As(V) exposure causes maximum decline in Ile (60%) level followed by Val (45%), Met (32%), Lys (18%), Thr (8%) and Leu (4%) in comparison to control (Fig. 6a). Likewise in shoots, As(V) exposure negatively affected amino acids *viz.*, Ile (80%), Val (64%), Met (32%), Thr (31%), Leu (27%) and Lys (24%) with respect to control (Fig. 6b). On contrary, Se(VI) supplementation to As(V) exposed seedlings improved the level of EAAs in roots with increase in the levels of Ile (373%), Lys (174%), Met (167%), Leu (112%), Thr (39%) and Phe (31%) as compared to As(V) alone. A similar response of EAAs was observed in shoots of the As(V)+Se(VI) exposed plants.

The level of EAAs (Met, Thr, Ile, Leu, Lys and Val) was declined under As(V) stress, while Se(VI) supplementation increased or reinstated their level in compared to their respective control or As(V) treated plants. The inhibitory effect of As(V) on EAAs contents may be ascribed to interruption in nitrogen metabolism and AA biosynthesis and degradation pathways (Finnegan and Chen 2012), whereas the beneficial effect of Se(VI) was mostly associated to lower As accumulation in plants. Moreover, Se intervened oxidative stress removal might have been attributed to the lower peroxidation of amino acids due to lesser free radicals. However, increased level of Phe during As(V) stress like cadmium stress (Zoghlami *et al.* 2011) may be used in the activation of secondary metabolites such as phenolic compounds for metalloid detoxification through hydrogen-donating and radical scavenger properties (Dixon and Paiva 1995).

The non-essential amino acids (NEAAs) *viz.*, Arg, Tyr, Asp, Ser, His, Glu, Pro, Gly, Cys and Ala responded in tissue and metalloid specific manner. Stress responsive NEAAs *viz.*, Glu (245 and 178%), Gly (143 and 95%), Cys (147 and 249%), Pro (99 and 117%), His (115 and 30%) and Ala (97 and 89%) were found to be enhanced in As(V) treated root and shoot respectively (Fig. 6 a,b). However significant increase was also observed in these NEAAs during Se(VI) addition either in the presence or absence of As(V). Cysteine is a central metabolite for antioxidant defence system and detoxifies metal through GSH / phytochelatin synthesis.[46] Glu and Gly are also used for GSH and phytochelatin synthesis (Sharma and Dietz 2006). In the present study, the increase in these amino acids during As(V) and Se(VI) exposure is associated with their utilisation in the phytochelatin synthesis. However, His increased level during metalloid stress verified by an arsenite-inducible, cysteine- and histidine-rich RNA-associated heat shock protein named AIRAP, seems to be induced by As(III) exposure (Sok *et al.* 2001). Similar to the present study, an induced level of proline content in rice was noticed upon As(III) exposure (Mishra and Dubey 2006), which imparts its defensive role towards reactive oxygen species and also works as osmoprotectant (Taylor 1996).

Some of the NEAAs such as Arg (62 and 31%), Ser (86 and 40%) and Asp (44 and 64%) decreased in As(V) alone treated roots and shoots. However, Se(VI) supplementation during As(V) stress recovered their level to some extent than As(V) alone treatment.

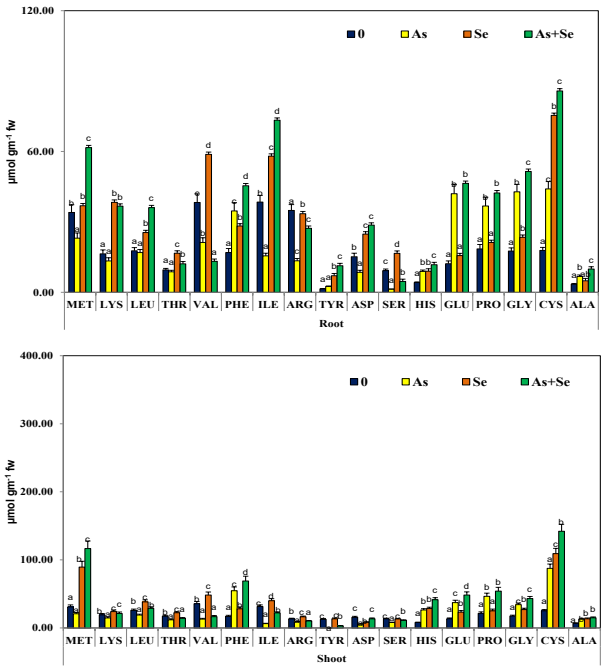

Fig. 6 (a, b): Amino acid profile in root (a) and shoot (b) of the rice seedlings after 15 d of treatments (0, 2.5 µM Se, 50 µM As and 50 µM As + 2.5 µM Se). All the values are means of triplicate ± S.D. ANOVA significant at $p \leq 0.01$. Different letters indicate significantly different values between treatments (DMRT, $p \leq 0.05$).

Depleted level of Ser during As(V) stress entails its utilisation in cysteine biosynthesis. Selenium addition ameliorated As(V) stress, leading to increased level of these AAs upto control or higher than that. Thus, addition of Se(VI) exhibited the its affirmative role on amino acid metabolism either alone or in presence of As(V).

Conclusion

Exposure of As to the rice plants causes phytotoxicity whereas exogenous application of se ameliorate the As induced toxicity in rice plants. Supplementation of Se improves the growth as well as essential amino acids and recovered the damage caused by As induced oxidative stress. Selenium supplementation reduced the cellular As buildup resulting in reduced phytotoxicity symptoms predominantly in roots than shoots of rice plant although SAT, γ-GT and CS activities were higher in shoots than roots. Phytochelatin synthase activity and phytochelatin concentrations were higher in root than shoot of rice plants during Se supplementation during As exposure compared to As and Se individual exposure. It seems that the combined exposures of As and Se boosted PC concentration including higher activities of γglutamyl cysteine synthase activity (γECS).

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Kumar, A., Singh, P. K., Srivastava, S.

SUPPLEMENTARY INFORMATION

Materials and methods

Photosynthetic pigments estimation

For estimation of photosynthetic pigments, plant material (300mg) was ground in chilled 80% acetone in dark. After centrifugation at 10 000g for 10 minutes at 4 °C, absorbance of the supernatant was taken at 480, 510, 645 and 663 nm. The content of chlorophylls was estimated by the method of Arnon (1949) and that of carotenoid content by using the formula given by Duxbury and Yentsch (1956).

Cysteine, non-protein thiols and glutathione measurements

The cysteine content in plant material was estimated by using acid–ninhydrin reagent (Gaitonde, 1967). The non-protein thiol (NPTs) content was measured by following the method of Ellman (1959). The level of GSH and GSSG was measured by following the protocol of Hissin and Hilf (1976).

Detailed methodology for amino acid analysis

The pico tag method (Bidlingmeyer *et al.* 1984) was followed for estimation of amino acids on Waters – HPLC system. 200 mg of homogenized rice plant samples were hydrolyzed in 10 mL 6N HCl in an oven for 12 hours at 120°C. 10µl of filtered hydrolyzed samples and of standard (2.5 µmoles/mL in 0.1 N HCl) were derivatised with phenylisothiocynate in vacuum oven at 55°C for 30 minutes at 75 milli torr after three rounds of drying and redrying. The derivatives samples were then diluted with pico tag sample diluent and filtered with syringe filters. 20 µl of this was then injected into the system. Chromatographic analysis of the extracts was performed with a Waters Binary gradient HPLC system with accessories module 2475, Waters, Milford, MA, USA equipped with a degasser (DG2), a binary pump module (515), Temperature control module (TC2), Pump control module (PC2) and a photodiode array detector (Waters 2998) (Waters origin USA, Ireland, Singapore, Malaysia, Australia). The separation was carried out at room temperature using a Pico Tag amino acid C18 column (3.9 X 15 cm; 5 µm). For each sample, 20 µl of extract was injected and the column was eluted at 1mL min^{-1}, with an optimized gradient established using solvents A (940 mL of 0.14 M sodium acetate, pH 6.40, containing 0.05% triethylamine and 6% acetonitrile) and B (60% acetonitrile in water). A step-by-step gradient was used with an increase of proportion of solvent B until it reached 46% during 10 minutes, followed by an increase upto 100% in 5 minutes, with a flux of 1 mL min^{-1}. The column was then cleared and optimised to 100% A for 8 minutes at 1 mL min^{-1}.

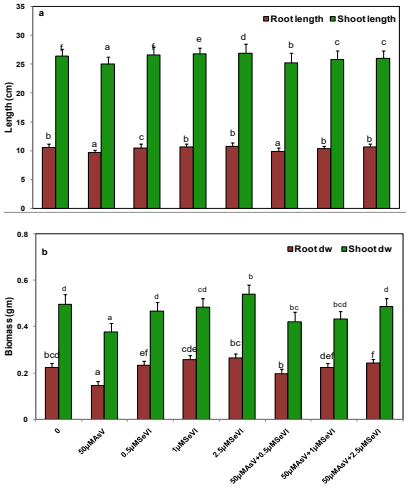

Fig. S1 (a, b): Changes in length (a) and dry weight (b) of rice root and shoot after 15 d of treatments (0, 50 µM As, 0.5 µM Se, 1 µM Se, 2.5 µM Se, 50 µM As + 0.5 µM Se, 50 µM As + 1 µM Se, 50 µM As + 2.5 µM Se). All the values are means of 5 replicate (n=5) ± S.D. ANOVA significant at $p \leq 0.01$. Different letters indicate significantly different values between treatments (DMRT, $p \leq 0.05$).

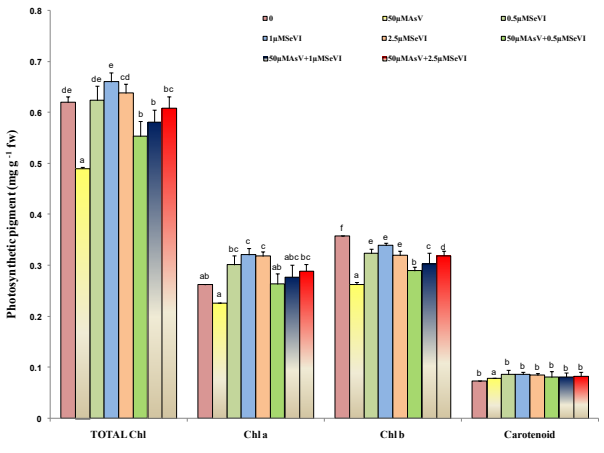

Fig. S2: Changes in photosynthetic pigments; chlorophyll a, chlorophyll b, total chlorophyll and carotenoid of the rice seedlings after 15 d of treatments (0, 2.5 µM Se, 50 µM As and 50 µM As + 2.5 µM Se). All the values are means of 3 replicate (n=3) ± S.D. ANOVA significant at $p \leq 0.01$. Different letters indicate significantly different values between treatments (DMRT, $p \leq 0.05$).

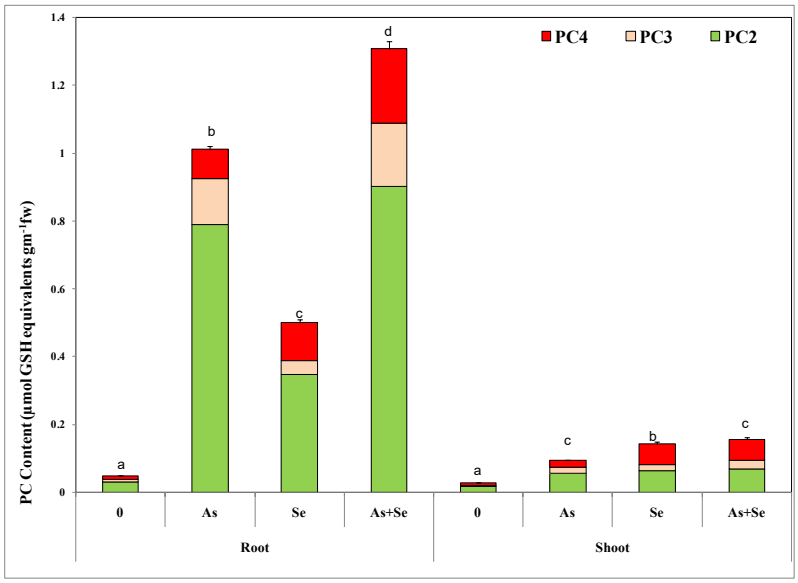

Fig. S3: Changes in the level of total PCs in roots and shoots of the rice seedlings after 15 d of treatments (0, 2.5 µM Se, 50 µM As and 50 µM As + 2.5 µM Se). All the values are means of 3 replicate (n=3) ± S.D. ANOVA significant at $p \leq 0.01$. Different letters indicate significantly different values between treatments (DMRT, $p \leq 0.05$).

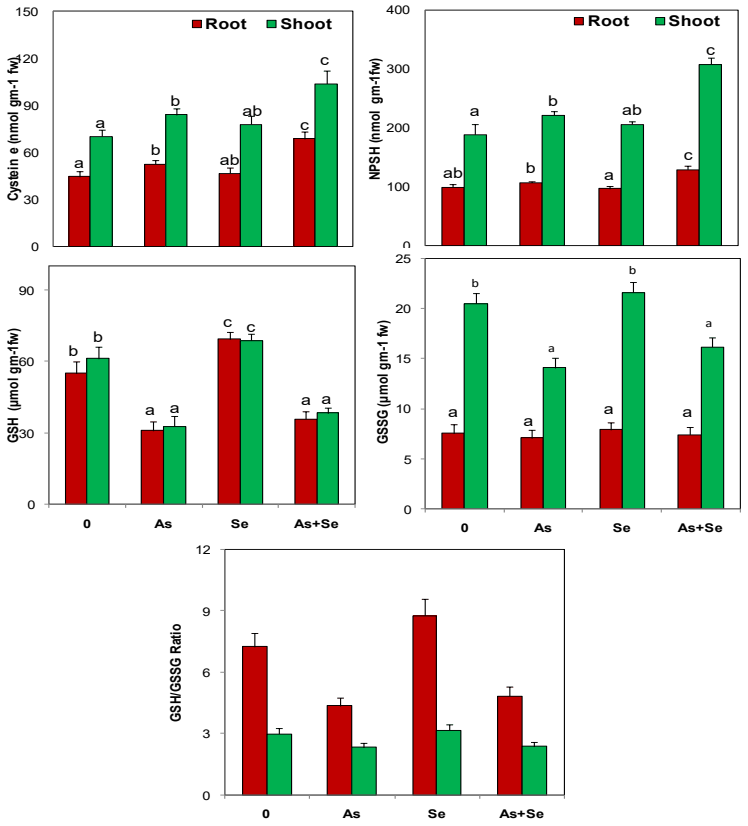

Fig. S4: Changes in cysteine (a), NPSH (b), GSH (c), GSSG (d) and GSH/GSSG (e) in roots and shoots of the rice seedlings after 15 d of treatments (0, 2.5 µM Se, 50 µM As and 50 µM As + 2.5 µM Se). All the values are means of 3 replicate (n=3) ± S.D. ANOVA significant at $p \leq 0.01$. Different letters indicate significantly different values between treatments (DMRT, $p \leq 0.05$).