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Research Article

Selenium elicitation and increase of secondary metabolites production by suspension-cultured *Astragalus verus* cells

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Abstract

Secondary metabolites with low molecular weight and high economic, pharmaceutical and industrial values play an important role in protecting plants against biotic and abiotic stresses. Astragalus is a medicinal herb, rich in secondary metabolites. Selenium (Se) is an essential element for humans, animals, many bacteria, as well as a beneficial element for plants. The plants response to selenium is depending on their species and the Se supply concentration. In the present study, calli were established from the seeds of *Astragalus verus* and suspension cultures were established from the calli. The cells were treated with different concentrations of sodium selenate (0, 0.5, 2.5, 12.5, and 62.5 µM) for 1 week at their logarithmic growth phase (based on growth curve). Cell growth and viability significantly decreased by selenium at concentrations higher 2.5 µM. In comparison with control group, significant increases in Abscisic acid and Salicylic acid were observed at higher Se concentrations, whereas methyl jasmonate significantly increased at lower Se supply. At 62.5 µM Se supply the contents of alkaloids, phlobaphene, steroidal and triterpenoid saponins, and tannin respectively increased by 5.1, 1.51, 1.62, 1.75, and 2.04 times of control. The results provides a practical method for remarkable stimulation of natural compounds of Astargalus by Se.

Keywords: Alkaloids, *Astragalus verus*, Phytohormones, Phlobaphene, Saponin, Sodium selenate

Introduction

Astragalus is the largest genus of flowering plants of Fabaceae with antioxidant, anti-hypertensive, anti-viral, anti-cancer, anti-inflammatory, and anti-diabetic properties (Li *et al.*, 2014). Having divers and valuable secondary metabolites e.g., triterpenoid and steroid saponins, phenylpropanoids, and alkaloids makes it to be regarded as an important medicinal plant (yang *et al.*, 2013). Some species of Astragalus can absorb and accumulate high concentrations of Se and thus are toxic for herbivorous animals (Maassoumi, 2016).

Selenium (Se) is a metalloid and an essential micronutrient for humans, animals, many bacteria, and a beneficial element for plants. It is found in nature in elemental selenium, selenide, selenite and selenate forms. Selenate enters into the plant via sulfate transporters while selenite is absorbed through phosphate transporters.

There are some literatures reporting protection role of Se for plants against abiotic stresses such as cold, drought and heavy metals (Gupta and Gupta, 2017). Reducing uptake, chelation and detoxification of heavy metals, and decrease of reactive oxygen species (ROS) through promotion of antioxidants have been suggested as the mechanisms of Se function in stressed plants (Alves *et al.*, 2020). Studying the physiological effects of Cr toxicity on *Brassica juncea*, Handa *et al.* (2018) have found that application of Se protected the plants by restoring growth, and defense systems through enhancement of secondary metabolites (Handa *et al.*, 2018). Also, treatment of *Melissa officinalis* with Se had positive effects on plant biomass, ascorbic acid, protein, and certain secondary metabolites e.g., caryophyllene, and caryophyllene oxide (Tavakoli *et al.*, 2020).

It should be noted however that the beneficial effect of Se is dose-dependent, and at higher concentrations it may adversely affect the plant metabolism, partly because of its competition with sulfure which results in lower sulfate uptake by the plant, and partly because of production of H_2O_2 (Antunes and Brito, 2017; Alves *et al.*, 2020).

Xue and co-workers (1993) revealed that seleno-compounds can quench superoxide anions and hydroxyl radicals directly. Also, it has been reported that Se can induce ROS scavenging through a non-enzymatic dismutation of superoxide anions to form H_2O_2 (Cartes *et al.*, 2010).

In spite of being a very simple molecule, H_2O_2 has a key role in cellular signaling, by oxidative modulation of the activity of redox sensitive proteins. It also affects

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the production of defensive secondary metabolites through regulation of expression and activities of defense-related genes (Samari *et al.*, 2020; Kahromi and Khara, 2021). On the other hand, changing H_2O_2 homeostasis activates cellular sensing mechanisms and hormonal signaling pathways to integrate wide range of physiological processes. Phytohormons serve as the key endogenous factors in mediating plant stress response (Golldack *et al.*, 2013; Verma *et al.*, 2016).

A few literature is available about the effects of Se on plant hormones and secondary metabolites (Handa *et al.*, 2018; Tavakoli *et al.*, 2020). To the best of our knowledge however, effect of Se have not been studied on *Astragalus verus* yet. In the present study, the effects of different concentrations of Se on the production of secondary metabolites by suspension-cultured Astragalus cells were investigated. In addition, the underlying mechanism(s) of their production in relation to phytohormones and H_2O_2 were discussed.

Materials and methods

Cell culture, treatment, biochemical, and physiological studies: Seeds of *A. verus* were obtained from General Department of Natural Resources and Watershed (Isfahan-Iran). The seeds were surface-sterilized and placed on modified LS media, pH 5.8, and solidified with 0.8% agar (Rezaei *et al.*, 2011). The calli were appeared from seed endosperm after 7-12 days and were sub cultured every two weeks. Suspension cultures were established from the calli in the above mentioned medium without agar. The cells were incubated in dark at 25 °C on reciprocal shaker at 120 rpm, and were sub-cultured every 7 days.

On day 7 (mid of logarithmic growth phase) Se in the sodium selenate form was filter-sterilized and then added to the cell to the final concentrations of 0, 0.5, 2.5, 12.5, 62.5 and 312.5 µM. The cells were harvested after one week, thoroughly washed, and divided to two parts. The first part was subjected to measurement of viability and fresh weight. The second part was fixed by liquid N_2 and kept at -80 °C for biochemical analysis. Since the 312.5 µM Se was fatal for the cells, lower concentrations of Se were selected for analyzes.

Determination of cell viability was performed using Evans blue (Rezaei *et al.*, 2011).

In order to measure H_2O_2 content, the samples were extracted with 0.1% trichloroacetic acid (TCA). Subsequently, 10 mM potassium phosphate buffer (pH 7.0) and 1 M potassium iodide were added to extract and was read at 390 nm by a UV– VIS spectrophotometer (Cintra 6, GBC, and Australia). The content was calculated based on a standard curve of 0-30 µM of H_2O_2 (Velikova *et al.*, 2000).

The lipid peroxidation of cell membrane was measured by quantitation of malondialdehyde (MDA) - thiobarbituric acid (TBA) complex. The absorbance was read at 532 nm and corrected for the non-specific absorbance at 600 and 400 nm for protein and sucrose, respectively Koobaz *et al.*, 2017). The amount of MDA was calculated using the following formula:

MDA (nM) = [(A532-A600) - (A440-A600)] × 0.0571×10^6 / 157000

The total contents of phenolics and tannins were determined by Folin-ciecalteu method, using gallic acid and tannic acid as standards, respectively (Makkar *et al.*, 1993).

Phlobaphene was extracted with butanol: HCl (70:30 v: v) followed by incubation at 37° C for 1h and centrifugation (12000 ×g, 15 min). Absorbance of the supernatant was read at 565 nm, and expressed as changes of absorbance per g FW (Wu *et al.*, 2020).

Anthocyanins were extracted with a mixture of MeOH: HCl (99:1), left overnight, read at 550 nm and quantified using an extinction coefficient of 33000 M^{-1} cm^{-1} (Krizek *et al.*, 1998).

Total flavonoid content was determined by Akkol *et al.* (2008) method. Flavonoid content was calculated based on the rutin standard curve.

For extraction of saponins, the cells were boiled three times and after filtration, the aqueous extract was concentrated and partitioned once with an equal volume of ethyl acetate. The ethyl acetate layer was further partitioned with an equal volume of *n*-butanol, which was subsequently evaporated to dryness. The dry crude powder was used as crude saponin extract. Total saponin content was determined by the vanillin-sulfuric acid method (Cheok *et al.*, 2014). A part of this extract was mixed with vanillin (8 %,w/v) and sulfuric acid (72 %, w/v), and then the mixture was incubated at 60° C for 10 min followed by cooling in an ice water bath for 15 min. Absorbance was measured at 544 nm to determine triterpenoid saponin content using oleanolic acid as standard. The second part of the extract was mixed with anisaldehyde, sulfuric acid and ethyl acetate. Absorbance of this solution was read at 430 nm in order to quantitate steroidal saponins, using diosgenin as standard.

In order to measure the total alkaloid, the samples were homogenized with 96% ethanol, and sequentially extracted with sulfuric acid 5%: Et_2O (1:1), and $CHCl_3$ as described by Marek *et al.* (2003). Total alkaloid content was calculated at 254 nm using ergotamine standard curve.

Radical scavenging capacity (RSC) of *A. verus* cells was measured by the method described by (N'guessan *et al.*, 2007).

For determination of phytohormones, the cells were extracted in 1 mL of absolute MeOH in an ice bath and were kept overnight. The extract was filtered by a sterile syringe filter (0.22 µm) and then injected to a

HPLC system (Waters e2695) on a C18 column (Perfectsil Target ODS-3 (5 µm), 250×4.6 mm; MZ Analysentechnik, Germany). Phytohormones were eluted at a flow rate of 1 mL min^{-1} with a gradient of MeOH: acidic water (deionized water containing 0.67% acetic acid, pH 3.0). Abscisic acid (ABA), salicylic acid (SA), and methyl jasmonate (MJ) were detected at 220 nm, and were quantified by comparison of their

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Table 1. Growth parameters and redox status of treated *A. verus* cells with different concentration of selenium

Growth parameters and redox status	Se treatment (µM)				
	0	0.5	2.5	12.5	62.5
Fresh weight (g)	5.5 ± 0.37 [a]	5.2 ± 0.27 [a]	5.4 ± 0.32 [a]	4.6 ± 0.04 [b]	3.7 ± 0.19 [c]
Viability (%)	100 ± 2.07 [a]	95.8 ± 5.51 [a]	93.2 ± 2.03 [a]	81.9 ± 1.50 [b]	55.8 ± 1.31 [c]
H_2O_2 content (µmol/ g FW)	2.8 ± 0.12 [b]	2.9 ± 0.10 [b]	3.0 ± 0.11 [b]	3.2 ± 0.09 [a]	3.3 ± 05 [a]
MDA content (nmol/ gFW)	0.09 ± 0.0 [c]	0.09 ± 0.01 [c]	0.09 ± 0.0 [c]	0.10 ± 0.01 [b]	0.13 ± 0.0 [a]

The values shown are the mean of 3 replicates ± standard deviation. Different letters indicate significant differences at the level of P≤0.05 based on Duncan test.

retention times and peak area with genuine standards (Sigma) (Soleimani *et al.*, 2019).

Statistical analyze: The experiments were performed in a complete randomized design with three independent replications. For statistical studies, SPSS software version 22 was used. Comparisons of the data was performed by Duncan test at the level of P≤0.05.

Results

Treatment of *A. verus* cells with Se at concentrations of 0.5 and 2.5 µM had no significant effects on growth and viability of the cells (Table 1), however at 12.5 µM and higher concentrations significantly decreased cell viability and biomass. At 62.5 µM Se the viability and biomass of the cells reduced to 32.7% and 44.2% of their controls, respectively (Table 1).

Likewise, the rate of membrane lipid peroxidation and the content of hydrogen peroxide of the cells significantly increased by 12.5 and 62.5 µM Se, compared to the control group (Table 1).

Supply of *A. verus* cells with 12.5 and 62.5 µM Se increased the total content of their phenolics, phelobaphen, and anthocyanin, compared to control cells (Figure 1a-c).

The content of tannin and flavonoids of the cells however, significantly increased when the cells were treated with 2.5 µM and higher concentrations of Se (Figure 1d-e).

In all of the treatments the content of triterpenoid saponins of the cells were higher than the steroid ones (Figure 2). The highest saponin content was detected when the *A. verus* cells were treated with 62.5 µM Se, where again triterpenoid saponins were 1.6 fold of steroid saponins (Figure 2).

Treatment of the cells with 0.5, 2.5, and 12.5 µM Se, significantly reduced their alkaloid contents, while 62.5 µM Se brought 1.81 fold increase in alkaloids (Figure 3).

Except for 0.5 µM, Se at higher concentrations significantly increased total radical scavenging capacity of the cells, compared to untreated cells (Figure 4).

Treating cells with 12.5 and 62.5 µM of Se significantly increased ABA and SA, compared to control cells (Table 2). The content of MJ of 0.5 and 2.5 µM Se treated cells was significantly higher than untreated cells (Table 2).

Discussion

The effect of Se as a beneficial micronutrient on plants is dose-dependent. In non-accumulator plants, at low concentrations, Se stimulates the growth and activities of antioxidant system. Treatment of *Brassica juncea* with Se increased the production of secondary metabolites and alleviated toxicity Cr (Handa *et al.*, 2018). Treatment of *Melissa officinalis* with high concentration of selenium (5 µM) increased z-citral, citral, and geranyl acetate contents while low Se concentration (0.2 µM) increased caryophyllene oxide content of essential oils. At this concentration of Se, ascorbic acid content of the plant was the highest (Tavakoli *et al.*, 2020)

At high concentrations, Se increases MDA and H_2O_2 content, causes chlorosis, necrosis, and even cell death (Gupta and Gupta, 2017; Silva *et al.*, 2018). In the present study, Se supply concentrations above 2.5 µM decreased the growth and viability of *A. verus* cells whereas it increased the content of MDA and H_2O_2.

H_2O_2 represents a key signaling molecule that connects the signaling pathways of multiple phytohormones (Cerny *et al.*, 2018). ABA, MJ, and SA play important roles in mediating plant defense response against abiotic stresses. Increase of H_2O_2 and SA of Se-treated *A. verus* cells activated their antioxidant system. Indeed, by increasing H_2O_2 content of the cells, metabolism of the cells were more directed towards the production of secondary metabolites, the non-enzymatic components of antioxidant system of the plants.

It is well documented that SA and MJ behave antagonistically, while the SA and ABA relationship is synergistic (Verma *et al.*, 2016; Zhao *et al.*, 2017). Increasing SA content of Se-treated cells synergistically enhanced ABA content but antagonistically reduced the content of MJ.

Se can affect nitrogen metabolism of plants through its effect on sulfur metabolism. Alteration in nitrogen assimilation is one of the profound consequences for the synthesis of all nitrogen-containing metabolites such as secondary metabolites e.g., phenylpropanoids and alkaloids (White, 2018).

Alkaloid contents of *A.verus* cells increased by Se as well. Although alkaloids protect plants against environmental stresses, they can be toxic to plants at high concentrations. Increasing of alkaloid content of *A. verus* cells at high concentrations of Se decreased their biomass and viability.

Phenolic contents of *A. verus* cells increased by Se. Increase of phenolics, tannin, phlobaphene, anthocyanins, and flavonoids in response to different

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Table 2. Phytohormone contents of *A. verus* cells treated with different concentration of selenium

Phytohormones	Se treatment (µM)				
	0	0.5	2.5	12.5	62.5
ABA (mg/ gFW)	0.20 ± 0.08 [b]	0.16 ±0.01 [b]	0.18 ± 0.00 [b]	0.40 ± 0.12 [a]	0.42 ± 0.0 [a]
SA (mg/gFW)	7.57 ± 0.09 [c]	6.4 ± 0.69 [c]	7.35 ± 0.28 [c]	12.50 ±1.10 [b]	15.46 ± 0.80 [a]
MJ (mM)	128.7 ± 6.2 [c]	155.8 ± 5.6 [a]	150.0 ± 7. 9 [b]	129. 7 ± 13.0 [bc]	46.9 ± 11.7 [d]

The values shown are the mean of 3 replicates ± standard deviation. Different letters indicate significant differences at P≤0.05 based on Duncan test.

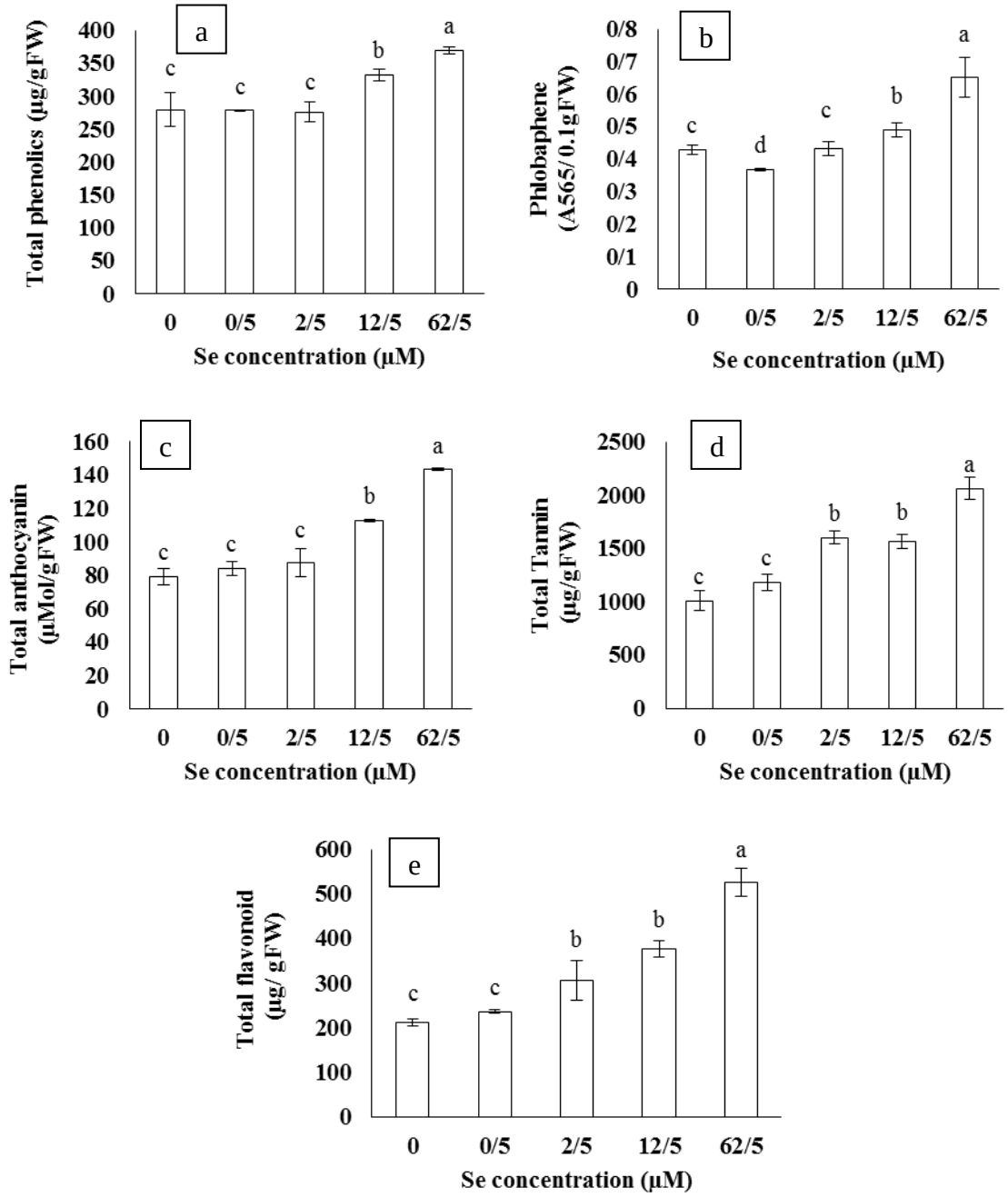

Fig. 1. Phenolic compounds of treated *A. verus* cells with different concentrations of selenium. Phenolics (a), phlobaphen (b), anthocyanin (c), tannin (d) and flavonoid (e) content. The values shown are the mean of 3 replicates ± standard deviation. Different letters indicate significant differences at P≤0.05 based on Duncan test.

signaling molecules were observed in high concentrations Se-treated *A. verus* cells. Phenolic compounds can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Zheng and Wang, 2001). As expected increase of these radical scavengers

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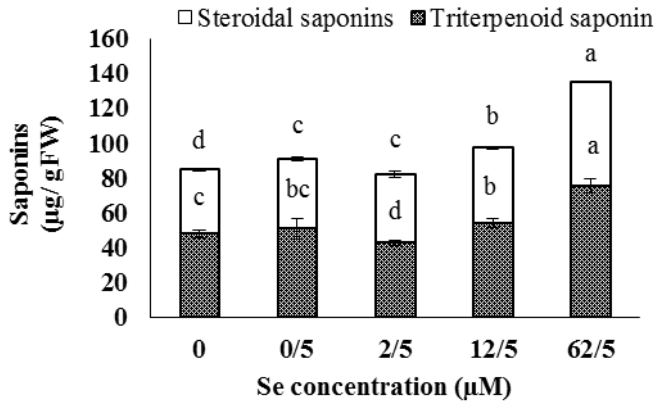

Fig. 2. Saponin content of treated *A. verus* cells with different concentrations of selenium. The values shown are the mean of 3 replicates ± standard deviation. Different letters indicate significant differences at P≤0.05 based on Duncan test.

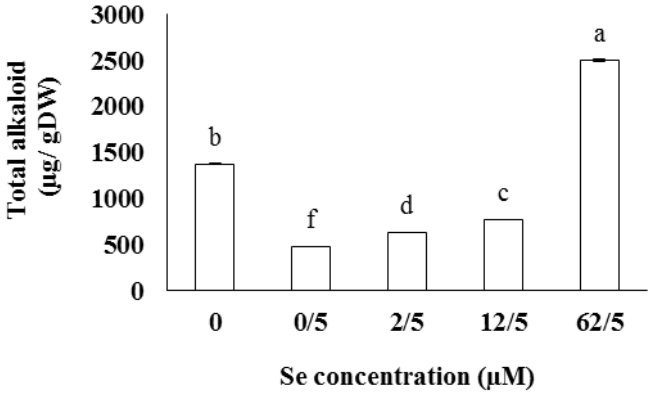

Fig. 3. Alkaloid content of treated *A. verus* cells with different concentrations of selenium. The values shown are the mean of 3 replicates ± standard deviation. Different letters indicate significant differences at P≤0.05 based on Duncan test.

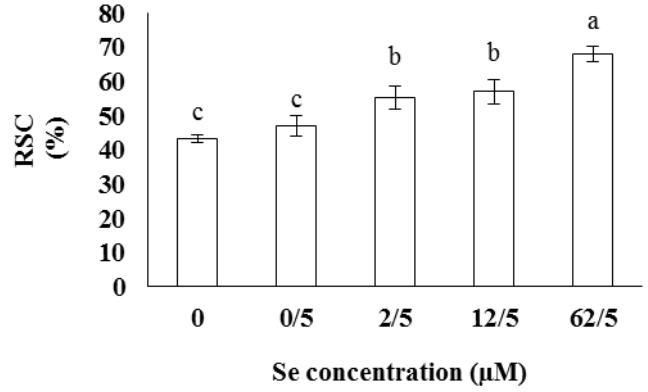

Fig. 4. Radical scavenging capacity of treated *A. verus* cells with different concentrations of selenium. The values shown are the mean of 3 replicates ± standard deviation. Different letters indicate significant differences at P≤0.05 based on Duncan test.

resulted in increase of RSC of Se-treated *A.verus* cells.

In the present study, along with increasing Se concentration in the media and following the enhancement of phenolics (including SA), the saponins and ABA contents also increased in the treated cells. Increase of saponins may be related to interaction SA and ABA. Increase of saponins in response to Se has been observed in *Panax ginseng* and capillary roots of *Psammosilene tunicoides* (Jeong and Park, 2006; Su *et al.*, 2021).

Conclusion

Selenium at low concentrations showed no significant effects on the metabolism of *A. verus* cells. However, At high concentrations however, elicited the cells and promoted signaling pathway resulting in production of secondary metabolite. These pathways were mediated by interactions among H_2O_2 and phytohormones i.e., SA, ABA, and MJ as signaling molecules.

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