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Role of Jasmonic and Salicylic Acid on Enzymatic Changes in The Root of Two *Alyssum Inflatum* Náyr. Populations Exposed to Nickel Toxicity

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Research Article

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Abstract

Salicylic acid (SA) and jasmonic acid (JA) as plant growth regulators (PGRs) have the potential to ameliorate plant development and tolerance to deleterious effects of toxic metals like nickel (Ni). Therefore, the current study was carried out to evaluate SA and JA's interactive effect on the root antioxidative response of two *Alyssum inflatum* Nyár. populations against Ni-toxicity. Two *A. inflatum* species under Ni-stress conditions (0, 100, 200, and 400 µM) were exposed to alone or combined levels of SA (0, 50, and 200 µM) and JA (0, 5, and 10 µM) treatments. Results showed that high Ni doses reduced the roots fresh weight (FW) in two populations than control; however, the use of external PGRs had ameliorated roots biomass by mitigated Ni-toxicity. Under Ni toxicity, SA and JA, especially their combination, induced high Ni accumulation in plants' roots. Moreover, the application of SA and JA alone, as well as combined SA + JA, was found to be effective in the scavenging of hydrogen peroxide (H_2O_2) by improving the activity of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) in both populations under Ni-toxicity. Overall, our results manifest that SA and JA's external use, especially combined SA + JA treatments, ameliorate root biomass and plant tolerance by restricting translocation Ni to the shoot, accumulating in roots, and also enhancing antioxidant defense systems.

Introduction

In the past decades, rapid mechanization gives rise to environmental pollution by heavy metals, which is considered an essential universal concern currently (Ramírez et al. 2021). A considerable amount of this pollution, such as nickel (Ni), has been entered the environment via the activities of both anthropogenic and natural origins (Ali et al. 2019). However, Ni as trace elements exists in natural soils, except in serpentine soils (Nagajyoti et al. 2010). Besides, Ni is an essential microelement (in the lower amount of 0.05–10 mg kg^{-1} dry biomass) for plant growth. Naturally, it exists in the structure of some metalloenzymes, including urease, glyoxalase-I, and a few superoxide dismutase (SOD), required for nitrogen metabolism in higher plants (Shahzad et al. 2018; Hassan et al. 2019; Leškova et al. 2020). However, exceeding the accumulation of Ni in plant tissues leads to toxicity in the plant and can negatively affect plenty of the plant's physiological and biochemical processes (Kosakivska et al. 2021). High Ni concentrations indirectly cause increased reactive oxygen species (ROS) production in plant cells (Silva et al. 2020). The high ROS generation in plant cells caused redox imbalance, disrupt enzyme activities, damage the structure of lipids and proteins, oxidative stress, and eventually cell death (Chaki et al. 2020). Subsequently, plant cells trigger antioxidant mechanisms to tolerate Ni-toxicity and combat oxidative stress (Amjad et al. 2019). For instance, excess Ni concentration in mustard plants (Abd_Allah et al. 2019), *Brassica juncea* (Khan et al. 2020), and *Solanum lycopersicum* L. (Jahan et al. 2020) leads to reduced growth traits by photosynthesis inhibition, the nutrients' absorption disruption, and oxidative stress due to ROS generation. Thus, they triggered antioxidant defense mechanisms to overcome the Ni-induced oxidative stress, including improving the activity of the antioxidant enzymes such as SOD, catalase (CAT), and ascorbate peroxidase (APX) as well as raising proline levels as a non-enzymatic antioxidant compound.

Likewase, when plants are exposed to abiotic stresses such as heavy metal, they use various stress tolerance strategies, including regulating the levels of endogenous hormones and their interaction pathways (Emamverdian et al. 2020). Plant endogenous hormones play a vital role in regulating responses to an extended range of internal and environmental stimuli (Mubarik et al. 2021). Jasmonic acid (JA) and salicylic acid (SA), arbitrated signaling pathways have been shown as potential tools in increasing plant tolerance to stress conditions (Caarls et al. 2015). Jasmonates [JA and methyl jasmonate (MeJA)] are considered endogenous-growth substances that play a significant role by modulating signal transduction mechanisms under stress conditions in numerous plant species (Raza et al. 2020). Many studies have been shown that JA and MeJA supplementation can recover the toxic effects of heavy metals in plants that have been described in diverse plants such as like soybean (*Glycine max*) (Keramat et al. 2009) and *Mentha arvensis* (Zaid and

Mohammad 2018) under high cadmium (Cd) concentration, and *Lemna valdiviana* under arsenic (As) toxicity (Coelho et al. 2020).

Likewise, SA is an important signaling molecule contributing to the fine-tuning of multiple physiological processes in plants. It is known as a stress messenger involved in abiotic stress signaling, as a plant defense responds to heavy metal toxicity (Zaid et al. 2019; Rhaman et al. 2020). Numerous studies show SA has a role in dropping the heavy metals toxicity in plants. For instance, a study by Wang et al. (2009) indicated that SA supplementation in maize (Zea mays L.) reduced the toxic effect of Ni by increasing APX and SOD activities and decrease hydrogen peroxide (H_2O_2) contents. Exogenously applied SA remarkably improved antioxidative enzymes' activities in *Lemna minor* under cadmium (Cd) toxicity (Lu et al. 2018) and *Nymphaea tetragona* (Gu et al. 2018), *Sorghum bicolor* L. under chromium (Cr) toxicity (Sihag et al. 2019), *Brassica campestris* under lead (Pb) toxicity (Hasanuzzaman et al. 2019), and watermelon plants under boron (B) stress (Moustafa-Farag et al. 2020).

The adjustment of plant defense mechanisms against biotic and abiotic stresses is determined by altering the concentration ratio of various interacting endogenous hormones, also the effect of each hormone on the endogenous concentration of others (Avalbaev et al. 2016; Mubarik et al. 2021). It is well-identified that the antagonistic effect has between the JA and SA cross-talk in various plants against biotic stress. However, there is a JA-SA cross-talk synergistic effect against biotic stress in rice (*Oryza sativa* L.) (Tong et al. 2012; Liu et al. 2015). Consequently, the adjustment of JA-SA signaling pathways' interaction may be critical for particular defensive responses in plants (Liu et al. 2015). According to our best knowledge and the previous report, no studies have reported SA/JA's interactive effects in plants exposed to heavy metal toxicity (Najafi Kakavand et al. 2019).

The species of *Alyssum inflatum* Nyár. species belong to the Brassicaceae family, is the native plant of serpentine and non-serpentine soil of west of Iran (Ghaderian et al. 2007). Besides, *A. inflatum* is considered as a Ni-hyperaccumulator plant (can accumulate Ni > 1000 $\mu g.g^{-1}$ in their dry biomass of leaves) (Reeves et al. 2018; Mohseni et al. 2018). Therefore, the current study focuses for the first time on the beneficial effects of JA and SA at different concentrations on antioxidant enzyme activities of roots of two metallicolous (M) and non-metallicolous (NM) *A. inflatum* species from serpentine and non-serpentine soils under several Ni doses.

Materials And Methods

Plant material and treatment

A. inflatum (M) seeds, is serpentine soils endemic population, were gathered from Marivan in the west of Iran, and *A. inflatum* (NM) seeds, non-serpentine soils population, were harvested from Shahu area in Kermanshah, Iran (Ghaderian et al. 2007; Najafi Kakavand et al. 2019). Overall, 108 pots for each population (a total of 216 pots for both populations) were used to perform this experiment. Originally, each replicate contained eight seeds of *A. inflatum* in a pot (450-ml) filled with perlite: sand (2:1) mixture individually. The pots were irrigated daily with tap water till germination and subsequently with Hoagland's solution as described by Najafi kakavand et al. (2019). All *A. inflatum* seedling was grown in a growth chamber environmentally controlled conditions (25 – 20°C, 16/8 h light/dark cycle, PPFD of 140 $\mu mol\ m^{-2}\ s^{-1}$), and the nutrient solutions were replaced by each 5-days. After six weeks of germination, the 108 pots related to each population were randomly divided into 36 groups, and each group received one treatment (n = 3 pots for each treatment). Four doses of Ni and three doses of SA and JA were exerted by applying various solution concentrations of Ni (0, 100, 200, and 400 µM), SA (0, 50, and 200 µM), and JA (0, 5, and 10 µM). Treatments were performed as a mixture of Ni and SA with Hoagland solution and JA as a foliar spray for three weeks. Plants were harvested 21 days after exposure, and then divided into shoot and root, and roots fresh weight (FW) were measured. Eventually, roots material was kept at − 70 ℃ for further examination.

Nickel contents evaluation

Nickel concentration was measured according to Ghasemi et al. (2009). Briefly, Ni concentrations of roots were determined by digesting 50 mg of dried material of roots in 2 ml of nitric acid (65%) overnight, followed by incubating at 90°C for 4 h. Subsequently, 1 ml H_2O_2 (30%) was added to the samples, and the mixture was heated again at 90°C until the digests had clarified. The volume of samples was adjusted to 10 ml with distilled water. Finally, the samples were analyzed for Ni concentration measurement by atomic absorption spectrophotometry (ASS 6200, Shimadzu, Japan).

Measurement of H_2O_2 concentration

Hydrogen peroxide (H_2O_2) contents were measured according to the Sergiev et al. (2000) method. Briefly, H_2O_2 was extracted from 50 mg fresh root materials by homogenization with 5 ml of cooled trichloro-acetic acid (TCA) [0.1% (W/V)]. The homogenates were centrifuged at 12000xg (15 min, 4°C). To measurement, the H_2O_2 content, the upper solution (0.5 ml) was mixed with 0.5 ml phosphate buffer (200 mM, pH 7.0) and 1ml potassium-iodide (1 M), and the mixture absorbance was recorded at 390 nm.

Extraction and Measurement of Enzyme activities

The root extraction was produced by homogenizing fresh root material (100 mg) with phosphate buffer (50 mM; pH 7.5) consisting of 1mM ethylene-diamine-tetraacetic acid (EDTA) and polyvinylpyrrolidone (PVP) [% 1(W/V)], and afterwards centrifuged (13,000×g; 15 min; 4°C). The upper solution was kept for enzyme activity assays.

The superoxide dismutase (SOD) activity was assayed using Giannopolitis and Ries (1977) method by measuring a decline in nitro-blue-tetrazolium (NBT) photo-reduction at 560 nm. The reaction solution (3 ml) consisted of 50 µl of enzyme solution, phosphate buffer (50 mM; pH 7.8), 13 mM methionine, 0.2 mM riboflavin, 1 mM NBT, 10 mM EDTA.

The catalase (CAT) activity was assayed using Aebi (1983) process, the process of catalase (CAT) activity was assayed. The assay solution included 800 µl of phosphate buffer (50 mM; pH 7.0), 100 µl of 30mM H_2O_2, and 100 µl of enzyme solution. The disintegration of H_2O_2 was monitored by a drop in absorbance at 240 nm. The enzyme activity was stated as U mg^{-1} protein.

The peroxidase (POD) was examined by the technique of Chance and Maehly (1955). The reaction solution consisted of 100 µl enzyme solution, phosphate buffer (200 mM; pH 7), guaiacol (1%), and 1mM H_2O_2. The oxidation of guaiacol was monitored at 470 nm using an extinction coefficient of 26.6 $mM^{-1}cm^{-1}$.

The ascorbate peroxidase (APX) activity was determined according to Nakano and Asada (1981) method. The APX assay included enzyme solution (50µl), phosphate potassium buffer (50 mM; pH 7), 1 mM EDTA, 5 mM ascorbate, and 1mM H_2O_2. The oxidation of ascorbate was monitored at 290 nm using an extinction coefficient of 2.8$mM^{-1}cm^{-1}$. Finally, the enzyme activity for all enzymes examined was stated as U. mg^{-1} protein.

The total protein in the roots was measured based on the method of Bradford (1976). Protein contents were measured according to a standard curve made using Bovine-serum albumin.

Proline concentration

Proline levels in the roots were estimated based on the Bates et al. (1973) method. Initially, dry roots materials (50 mg) were extracted with 10 ml 3% sulfosalicylic acid, and the mixture was centrifuged (1700 g; 10 min). Then, to 0.5 ml of upper solution, 0.5 ml glacial acetic acid and 1 ml ninhydrin reagent was added and held in a water bath (100°C) for 1 hr. Finally, the reaction was terminated by placing the sample tubes into a cold bath. The product was extracted by combining

1 ml of toluene to the reaction solution by vortexing for 1 min. Finally, the upper phase's absorbance is recorded at 520 nm, and proline concentrations are calculated from a standard graph of L-proline.

Statistical analysis

The GLM procedure of SAS was exerted for statistical analysis of the variance (one-way ANOVA) of each data set, and Duncan's test (at $P \leq 0.05$) was exerted to compare the mean values of treatments. For a better understanding of treatment effects, data analysis of variance was performed in three separate groups. In one group, all Ni and SA levels were considered, while in another group, analysis of variance was performed on all Ni and JA levels treatments. The third group of analyses included the three-way effects of Ni, SA, and JA. In this group, SA0 and JA0 were also used in all Ni levels to perform the analysis of variance with 20 treatments. The data stated are mean ± SD and n = 3. Also, principal component analysis (PCA) was done applying the fviz-pca function of the factoextra R package ver. 1.0.7 (Kassambara 2017) to visually biplot treatments and variables.

Results

Root fresh weight

At 100-µM and 200-µM Ni-treatment, root fresh weight (FW) of both M and NM plants did not indicate significant differences with control plants ($p \leq 0.05$). In contrast, root FW declined by 19.8% and 35.5% at 400-µM Ni concentration in M and NM species of *A. inflatum*, respectively, compared to control plants (Fig. 1a, b). Application of 200-µM SA on the plants' exposure to 400-µM Ni, roots FW incremented 1.26-and 1.85-times in M and NM species respectively, compared to only 400-µM Ni-treated plants. Similar results were obtained with 10-µM JA in plants under 400-µM Ni-toxicity so that a 1.28-fold increase in root FW of NM plants was observed in comparison to 400-µM Ni treatment. Application of 200-µM SA + 10-µM JA on M population increased the roots FW by 11.74% under 400-µM-Ni stress than control plants. Conversely, on the other hand, supplementation of 50-µM SA + 5-µM JA on the NM population enhanced the root FW by 7.25% with 400-µM-Ni stress relative to the controls (Table 1).

Table 1
Influence of combined SA + JA on Ni contents, FW, protein levels, H_2O_2 levels, and proline contents in roots of M/NM populations of *A.inflatum* exposed to Ni-toxicity

Treatments	Ni concentration (µg g^{-1} DW)		FW (g $plant^{-1}$)		Protein content (mg g^{-1} FW)		H_2O_2 content (µmol g^{-1} FW)		Proline content (µg g^{-1} DW)	
	M	NM	M	NM	M	NM	M	NM	M	NM
Control	50.9 ± 4.03^{q}	34.5 ± 3.74^{q}	0.90 ± 0.04^{fg}	0.66 ± 0.02^{opq}	5.47 ± 0.34^{nop}	6.87 ± 0.17^{i-l}	2.26 ± 0.16^{rs}	2.28 ± 0.10^{rs}	20.1 ± 2.01^{opq}	11.5 ± 1.17^{u}
(Ni0 + SA50 + JA5) µM	46.5 ± 4.14^{q}	46.4 ± 4.03^{q}	0.68 ± 0.02^{l-o}	0.54 ± 0.02^{tu}	4.77 ± 0.24^{pq}	8.15 ± 0.33^{h}	2.35 ± 0.14^{qr}	2.21 ± 0.03^{rs}	19.0 ± 2.13^{o-r}	15.6 ± 0.92^{t}
(Ni0 + SA50 + JA10) µM	51.8 ± 3.81^{q}	40.0 ± 5.56^{q}	0.67 ± 0.03^{nop}	0.62 ± 0.02^{qr}	5.68 ± 0.46^{mno}	8.26 ± 0.21^{hg}	2.73 ± 0.10^{k-o}	2.28 ± 0.03^{rs}	20.1 ± 1.67^{opq}	16.6 ± 0.36st
(Ni0 + SA200 + JA5) µM	51.9 ± 3.89^{q}	40.0 ± 4.58^{q}	0.81 ± 0.02^{h}	0.53 ± 0.04^{tu}	4.56 ± 1.10^{q}	6.76 ± 0.22^{i-l}	2.17 ± 0.07^{s}	2.25 ± 0.07^{rs}	25.8 ± 1.19^{jkl}	15.0 ± 1.51^{t}
(Ni0 + SA200 + JA10) µM	52.8 ± 3.18^{q}	47.1 ± 4.36^{q}	1.07 ± 0.01^{b}	0.38 ± 0.05^{w}	5.41 ± 0.64^{nop}	6.19 ± 0.20^{j-n}	2.51 ± 0.16^{pq}	2.22 ± 0.08^{rs}	23.2 ± 1.33^{m}	17.1 ± 0.98^{rst}
(Ni100 + SA0 + JA0) µM	602 ± 18.4^{k}	520 ± 19.3^{l}	0.86 ± 0.03^{g}	0.56 ± 0.04st	5.54 ± 0.34^{m-p}	9.43 ± 0.25^{def}	2.89 ± 0.21^{h-l}	2.66 ± 0.12^{m-p}	38.3 ± 0.73^{d}	23.2 ± 0.56^{m}
(Ni100 + SA50 + JA5) µM	797 ± 9.77^{i}	242 ± 16.0^{p}	0.72 ± 0.01^{kl}	0.65 ± 0.03^{opq}	5.23 ± 0.64^{opq}	10.4 ± 0.39^{bc}	2.82 ± 0.04^{i-m}	2.64 ± 0.11^{nop}	26.0 ± 1.45^{jk}	18.3 ± 1.25^{qrs}
(Ni100 + SA50 + JA10) µM	838 ± 19.7^{gh}	254 ± 25.5^{op}	0.76 ± 0.01^{ijk}	0.32 ± 0.01^{x}	6.58 ± 0.66^{i-l}	10.2 ± 0.28^{bcd}	2.73 ± 0.07^{l-o}	2.73 ± 0.05^{l-o}	23.9 ± 1.16^{klm}	16.6 ± 1.06st
(Ni100 + SA200 + JA5) µM	877 ± 28.8^{fg}	259 ± 24.0^{op}	0.97 ± 0.01^{de}	0.63 ± 0.01^{qpr}	5.74 ± 0.33^{mno}	8.16 ± 0.51^{h}	2.57 ± 0.07^{op}	2.70 ± 0.07^{mno}	27.1 ± 0.92^{ij}	20.6 ± 0.92^{op}
(Ni100 + SA200 + JA10) µM	895 ± 61.6^{f}	273 ± 23.0^{op}	1.16 ± 0.02^{a}	0.49 ± 0.04^{uv}	6.09 ± 0.49^{lmn}	7.29 ± 0.12^{i}	2.70 ± 0.04^{mno}	2.78 ± 0.06^{j-n}	27.3 ± 0.92^{ij}	23.8 ± 0.31^{lm}
(Ni200 + SA0 + JA0) µM	753 ± 8.05^{j}	745 ± 24.5^{j}	0.91 ± 0.03^{fg}	0.67 ± 0.05^{m-p}	6.58 ± 0.45^{i-l}	10.4 ± 0.33^{bc}	3.01 ± 0.14^{fgh}	3.01 ± 0.14^{fgh}	44.1 ± 1.46^{b}	27.6 ± 1.18^{ij}
(Ni200 + SA50 + JA5) µM	963 ± 24.9^{e}	288 ± 69.0^{no}	0.73 ± 0.03^{jk}	0.49 ± 0.01^{uv}	6.96 ± 0.38^{ij}	12.0 ± 0.66^{a}	3.03 ± 0.09^{fgh}	2.78 ± 0.12^{j-n}	28.6 ± 0.65^{hi}	21.1 ± 1.33^{no}
(Ni200 + SA50 + JA10) µM	976 ± 37.4^{e}	357 ± 12.5^{m}	0.77 ± 0.02^{hij}	0.67 ± 0.03^{nop}	6.29 ± 0.36^{j-m}	11.8 ± 0.48^{a}	2.91 ± 0.09^{hij}	2.81 ± 0.09^{j-n}	30.3 ± 0.73^{gh}	18.8 ± 0.88^{pqr}

Treatments	Ni concentration (µg g^{-1} DW)		FW (g $plant^{-1}$)		Protein content (mg g^{-1} FW)		H_2O_2 content (µmol g^{-1} FW)		Proline content (µg g^{-1} DW)	
	M	NM	M	NM	M	NM	M	NM	M	NM
(Ni200 + SA200 + JA5) µM	1034 ± 12.7^{d}	322 ± 22.5^{mn}	0.79 ± 0.01^{hi}	0.59 ± 0.02^{rs}	6.88 ± 0.50^{ijk}	10.5 ± 0.44^{b}	2.90 ± 0.08^{h-k}	2.95 ± 0.07^{hij}	31.6 ± 1.67^{fg}	26.0 ± 1.07^{j}
(Ni200 + SA200 + JA10) µM	986 ± 18.2^{e}	344 ± 27.0^{m}	0.93 ± 0.02^{ef}	0.47 ± 0.02^{v}	8.21 ± 0.26^{h}	9.67 ± 0.38^{cde}	2.88 ± 0.01^{h-l}	2.98 ± 0.05^{ghi}	33.4 ± 1.76^{ef}	27.3 ± 1.55^{ij}
(Ni400 + SA0 + JA0) µM	831 ± 29.1^{hi}	886 ± 19.5^{f}	0.72 ± 0.04^{klm}	0.42 ± 0.02^{w}	5.01 ± 0.55^{opq}	9.28 ± 0.36^{ef}	3.763 ± 0.12^{a}	3.64 ± 0.09^{ab}	54.6 ± 3.10^{a}	32.1 ± 0.96^{fg}
(Ni400 + SA50 + JA5) µM	1268 ± 16.2^{b}	743 ± 9.50^{j}	0.74 ± 0.03^{jk}	0.71 ± 0.03^{k-n}	5.07 ± 0.46^{opq}	11.6 ± 0.70^{a}	3.17 ± 0.07^{def}	3.00 ± 0.15^{gh}	35.6 ± 1.24^{e}	22.8 ± 1.39^{mn}
(Ni400 + SA50 + JA10) µM	1310 ± 39.8^{a}	818 ± 37.5^{hi}	0.93 ± 0.02^{ef}	0.53 ± 0.02^{tu}	4.55 ± 0.34^{q}	8.87 ± 0.47^{fgh}	3.14 ± 0.06^{efg}	2.92 ± 0.09^{hij}	34.6 ± 1.25^{e}	22.9 ± 1.04^{mn}
(Ni400 + SA200 + JA5) µM	1151 ± 37.3^{c}	803 ± 25.0^{hi}	0.99 ± 0.01^{cd}	0.57 ± 0.04st	6.70 ± 0.35^{i-l}	9.00 ± 0.63^{efg}	3.21 ± 0.188^{de}	3.48 ± 0.05^{bc}	41.8 ± 0.97^{c}	27.4 ± 1.68^{ij}
(Ni400 + SA200 + JA10) µM	1345 ± 16.9^{a}	900 ± 21.0^{f}	1.02 ± 0.01^{c}	0.55 ± 0.03st	6.17 ± 30.42^{k-n}	6.97 ± 0.96^{ij}	3.30 ± 0.11^{de}	3.32 ± 0.010^{cd}	39.4 ± 1.21^{d}	31.3 ± 1.58^{fg}

Data are introduced as a mean of each treatment group ± SE (n = 3). Different letters presented statistical differences in means by the Duncan test (at $p \leq 0.05$).

Root Ni concentration

After three weeks of treatment, M and NM populations of *A. inflatum* accumulated Ni significantly in the roots when treated with 400-µM Ni concentration (Fig. 2a, b). Salicylic acid application increased Ni accumulation by about 1.2-folds in both populations, compared to only 400-µM Ni-treated plants. Likewise, increasing JA concentration upsurges the Ni contents in the roots of the M population. Although, in NM plants treated with 400-µM Ni + 5-µM JA and 400-µM Ni + 10-µM JA, Ni levels were dropped by 12.3% and 7.33%, respectively, compared with Ni 400-µM treatment alone. Additionally, exogenous SA supply and foliar application of JA at the same time have positively affected the Ni-accumulation in the roots of both populations (Table 1). The average increase in root Ni contents, under 400-µM Ni + 200-µM SA + 10-µM JA treatment, was recorded as 38.2% in M and 1.50% in NM plants, concerning 400-µM Ni treatment.

Protein contents in roots

A moderate Ni (200-µM) dose increased the protein contents by 16.8% and 33.8% in M and NM species' roots, respectively, than control plants (Fig. 3a, b). With the SA and/or JA application, protein contents increased under 400-µM Ni stress in the roots of M/NM plants. Unlike this, the exogenous JA decreased the protein level in M populations' roots with an increase in Ni doses. Importantly, the external application of SA and JA in M/NM plants at the same time, increased the protein content, especially under 200-µM Ni stress. For example, the highest protein levels were observed in the simultaneous treatment of 200-µM Ni + 200-µM SA + 10-µM JA by 33.3% in the root of M population, and 200-µM Ni + 50-µM SA + 5-µM JA by 42.9% in the root of NM populations, compared to the root of control plants (Table 1).

Hydrogen peroxide level in root

On day 21 of 400-µM Ni-treatment, excised roots of M/NM plants demonstrated a significant accumulation of H_2O_2, which was not observed in control plants (Fig. 4a,b). Whereas the external application of SA and/or JA in plants exposed to Ni, we observed a declining trend in H_2O_2 content in roots of M/NM populations. Additionally, the simultaneous application of SA + JA in plants exposed to Ni showed a decreasing trend in H_2O_2 levels (Table 1). For instance, in the treatment of 400-µM Ni + 200-µM SA + 10-µM JA, the H_2O_2 content decreased by 12.2% and 8.22% in M/NM plants compared with plants treated with 400-µM Ni.

The activity of antioxidant enzymes

High doses of Ni indirectly lead to the generation of H_2O_2, which in turn stimulates the activity of antioxidative enzymes (SOD, CAT, POD, and APX) under Ni stress in M/NM plants (Fig. 5). However, the AXP activity in the roots of M-population exposed to high Ni doses did not significantly change compared to control plants (Fig. 5e,f). The highest Ni level (400-µM Ni) caused a maximal increment in the antioxidative enzyme activities, i.e., 51.6% and 59.2% (SOD), 44.1% and 22.4% (CAT) and 53.5% and 23.6% (POD) in M and NM plants, respectively, compared to the control plants. Likewise, the APX activity increased by 43.1% at 400-µM Ni concentration in NM plants, and there was no significant difference between these treatments compared to the control plants. The activities of four antioxidative enzymes in the roots of M/NM populations exposed to different Ni concentrations were changed in response to SA and JA alone and the simultaneous application of SA + JA. Percent of activities of SOD, POD, and CAT was remarkably improved with the application of SA and/or JA than the control plants in both populations. The highest enzyme activity, i.e., 58.1% (SOD), 57.3% (CAT), 21.0% (APX), and 61.9% (POD) were recorded in the roots of M plants by 400-µM Ni + 50-µM SA + 10-µM JA treatment whit respect to the control plants. In contrast, the highest enzymes activity, i.e., 69.0% (SOD), 53.9% (CAT), 48.8% (APX), and 35.8%(POD) in the roots of NM plants were observed under 400-µM Ni + 50-µM SA + 10-µM JA treatment whit respect to the control plants (Table 2).

Table 2
Influence of combined SA + JA on antioxidative enzymes activity in roots of M/NM populations of *A.inflatum* exposed to Ni-toxicity

	SOD activity (U mg^{-1}protein)		APX activity (U mg^{-1}protein)		POD activity (U mg^{-1}protein)		CAT activity (U mg^{-1}protein)	
Treatments	**M**	**NM**	**M**	**NM**	**M**	**NM**	**M**	**NM**
Control	516 ± 41.1st	513 ± 45.8st	31.9 ± 3.57^{mno}	45.3 ± 2.72^{ij}	376 ± 13.8st	473 ± 15.0^{l-p}	79.1 ± 3.84^{r}	117 ± 6.01^{h-l}
(Ni0 + SA50 + JA5) µM	664 ± 17.6^{n-q}	399 ± 34.9^{n}	38.8 ± 2.68^{kl}	52.8 ± 1.11^{gh}	415 ± 30.8^{p-s}	500 ± 31.5^{i-m}	84.8 ± 8.51^{pqr}	113 ± 4.74^{jkl}
(Ni0 + SA50 + JA10) µM	504 ± 60.8^{t}	495 ± 30.0^{t}	28.3 ± 3.29^{nop}	49.0 ± 4.62^{hi}	442 ± 47.4^{m-r}	408 ± 36.4^{q-t}	78.4 ± 11.2^{r}	130 ± 13.4^{fg}
(Ni0 + SA200 + JA5) µM	569 ± 63.9^{rst}	747 ± 102^{k-n}	39.7 ± 1.91^{k}	55.6 ± 2.45^{fg}	441 ± 52.0^{n-r}	395 ± 27.1^{rst}	84.1 ± 4.31^{qr}	114 ± 9.98^{jkl}
(Ni0 + SA200 + JA10) µM	627 ± 21.1^{o-r}	683 ± 81.2^{nop}	33.1 ± 3.56^{mn}	64.9 ± 0.69^{de}	454 ± 39.3^{m-q}	474 ± 21.6^{k-p}	79.3 ± 6.92^{r}	121 ± 8.50^{g-k}
(Ni100 + SA0 + JA0) µM	609 ± 12.4^{pqr}	719 ± 60.4^{lmn}	33.9 ± 2.52^{lm}	60.6 ± 4.65^{ef}	438 ± 13.5^{n-r}	526 ± 8.32^{h-l}	92.2 ± 0.85^{opq}	117 ± 4.77^{h-l}
(Ni100 + SA50 + JA5) µM	725 ± 25.8^{k-n}	572 ± 39.6^{rst}	33.5 ± 2.75^{m}	52.8 ± 2.26^{gh}	557 ± 60.7^{ghi}	471 ± 23.0^{l-p}	99.4 ± 8.13^{mno}	127 ± 7.82^{gh}
(Ni100 + SA50 + JA10) µM	562 ± 67.7^{rst}	738 ± 49.8^{k-n}	23.9 ± 1.07^{pqr}	51.7 ± 4.03^{gh}	475 ± 36.3^{k-p}	417 ± 23.9^{o-s}	96.6 ± 9094^{nop}	123 ± 2.08^{g-j}
(Ni100 + SA200 + JA5) µM	594 ± 23.9^{qrs}	896 ± 30.1^{ghi}	28.2 ± 3.84^{nop}	61.4 ± 4.41^{de}	572 ± 42.9^{gfh}	400 ± 13.5^{q-t}	95.9 ± 5.26^{n-q}	113 ± 4.03^{jkl}
(Ni100 + SA200 + JA10) µM	702 ± 32.0^{mno}	979 ± 23.5^{fg}	27.6 ± 2.79^{opq}	65.8 ± 2.60^{d}	569 ± 66.7^{gfh}	496 ± 30.6^{j-n}	97.6 ± 4.85^{mno}	112 ± 6.70^{jk}l
(Ni200 + SA0 + JA0) µM	797 ± 53.2^{jkl}	780 ± 21.8^{j-m}	27.9 ± 1.77^{opq}	65.3 ± 4.12^{de}	532 ± 35.1^{g-k}	540 ± 12.1^{g-j}	99.0 ± 6.56^{mno}	129 ± 2.90^{fgh}
(Ni200 + SA50 + JA5) µM	728 ± 66.0^{k-n}	713 ± 82.4^{l-o}	23.0 ± 1.63^{qr}	46.5 ± 0.81^{i}	587 ± 35.0^{gf}	440 ± 23.9^{n-r}	108 ± 15.1^{lmn}	109 ± 10.8^{klm}
(Ni200 + SA50 + JA10) µM	812 ± 47.2^{ijk}	730 ± 91.1^{k-n}	23.5 ± 1.37^{pqr}	54.8 ± 3.03^{g}	585 ± 52.4^{gf}	357 ± 13.5^{t}	121 ± 8.14^{g-k}	109 ± 15.4^{klm}
(Ni200 + SA200 + JA5) µM	784 ± 50.6^{j-m}	893 ± 35.9^{ghi}	21.8 ± 1.61^{r}	64.8 ± 2.96^{de}	573 ± 30.5^{gfh}	556 ± 23.0^{ghi}	114 ± 1.62^{i-l}	155 ± 4.76^{d}
(Ni200 + SA200 + JA10) µM	712 ± 62.9^{l-o}	1169 ± 31.3^{cd}	21.9 ± 1.81^{r}	62.4 ± 4.50^{de}	616 ± 20.5^{f}	531 ± 25.4^{g-j}	111 ± 6.87^{kl}	191 ± 7.87^{bc}
(Ni400 + SA0 + JA0) µM	1067 ± 21.6^{ef}	1257 ± 52.7^{bc}	32.5 ± 3.46^{mno}	79.7 ± 2.38^{b}	809 ± 29.7^{d}	619 ± 26.1^{f}	141 ± 4.37^{ef}	151 ± 5.85^{de}
(Ni400 + SA50 + JA5) µM	1154 ± 54.8^{de}	953 ± 110^{g}	40.4 ± 5.02^{jk}	65.2 ± 3.14^{de}	825 ± 31.7^{cd}	537 ± 32.0^{g-j}	160 ± 7.99^{d}	127 ± 6.49^{ghi}

	SOD activity (U mg^{-1}protein)		APX activity (U mg^{-1}protein)		POD activity (U mg^{-1}protein)		CAT activity (U mg^{-1}protein)	
(Ni400 + SA50 + JA10) µM	1233 ± 69.2^{bcd}	1268 ± 93.1^{b}	35.3 ± 1.41^{klm}	73.5 ± 5.00^{c}	987 ± 68.6^{a}	619 ± 32.74^{f}	185 ± 4.41^{c}	161 ± 7.95^{d}
(Ni400 + SA200 + JA5) µM	863 ± 20.4^{hij}	1241 ± 40.7^{bcd}	24.3 ± 1.48^{pqr}	62.4 ± 5.45^{de}	881 ± 39.5^{bc}	570 ± 41.2^{gfh}	129 ± 3.19^{fgh}	202 ± 5.15^{b}
(Ni400 + SA200 + JA10) µM	911 ± 42.1^{gh}	1657 ± 43.2^{a}	25.1 ± 1.54^{pqr}	88.7 ± 4.97^{a}	910 ± 57.7^{b}	737 ± 45.1^{e}	156 ± 6.77^{d}	255 ± 6.24^{a}

Data are introduced as a mean of each treatment group ± SE (n = 3). Different letters presented statistical differences in means by the Duncan test (at $p \leq 0.05$).

Root Proline levels

The contents of an important non-enzymatic antioxidant compound, proline, altered remarkably under Ni-toxicity. However, Ni toxicity increased the proline concentration in a dose-depended manner. The proline levels in the roots of both M/NM populations increased by 63.5% exposed to Ni 400-µM, compared to the control plants (Fig. 4c, d). However, exogenous SA and JA increased proline accumulation under high Ni doses in M/NM plants, compared to the control. Despite this, the use of exogenous SA and/or JA in the roots of both populations exposed to high Ni doses showed a decreasing trend of about 1.25-fold compared to 400-µM Ni-stress alone. Simultaneous treatment with SA and JA in both populations had improved Ni toxicity by increasing proline levels at 400 µM Ni. For example, the highest proline contents (51.7% and 63.2%) were observed in the simultaneous treatment of 400-µM Ni + 200-µM SA + 5-µM JA in the root of M population and 400-µM Ni + 200-µM SA + 5-µM JA in the root of NM species, respectively, compared to the root of control plants (Table 1).

Principal Component Analysis (PCA)

The responses of M and NM of *A. inflatum* populations to SA, JA, and SA + JA treatments under Ni-toxicity were assessed through the principal component analysis (PCA) score plot. The PCA results of *A. inflatum* in M and NM population, treated with SA under Ni toxicity, showed that the first two components explain 92.3% and 81.8% of the total variation, respectively (Fig. 6a, b). In the M population of *A. inflatum*, proline, CAT, POD, SOD, H_2O_2, and Ni concentration were positively correlated. In contrast, FW and protein showed a slight correlation with the mentioned traits and were negatively correlated with APX (Fig. 6a). In the NM population, a more positive correlation can be seen among the evaluated traits, but it is noteworthy that all of these traits are located in the sections where the high levels of Ni are also located, or in the other word, in the opposite direction to the control (Fig. 6b)

Biplot of *A. inflatum* in M and NM-population treated with JA under Ni toxicity revealed that the first two components explain almost 87.7% and 79.4% of the total variance. In both populations, the variables in this evaluation followed a similar pattern as SA treatment. All enzymes, H_2O_2, proline, and Ni, positively correlated with each other and strongly influenced the first component (Fig. 6c, d).

Considering the combination of SA + JA treatment under Ni toxicity, it was shown that the first two components explain 78.2% and 79.7% of the total variance in M and NM populations, respectively (Fig. 6e, f). The highest Ni accumulation in both populations' roots occurred after the combination of SA + JA treatments, which was associated with the highest enzymatic activity, proline accumulation, and H_2O_2.

Discussion

In the current study, the effect of JA and SA supplementation, alone and combined, was assessed for its capability to restrict Ni's toxic effect on the physiological and biochemical features of both M/NM populations of *A. inflatum* under Ni-

toxicity. High Ni accumulation in various plant tissues has toxic effects on various growth and physiological processes, such as inhibits growth and photosynthetic capability, leading to a decrease in biomass (Ghori et al. 2019; Sharma et al. 2020). The toxic effect of Ni on growth inhibition further enhances with an increment in high Ni levels (Hassan et al. 2019). The present study showed a reverse relation between Ni accumulation and root FW of the plant. By augmenting the Ni concentration to 400-µM, high Ni accumulation in the roots of two populations of *A. inflatum*, and alternatively, lead to a reduction in roots FW. Besides, it was found that the roots of plants suffer the most damage compared to the shoots, where the roots are exposed to the highest doses of Ni, which has reverse effects on root growth and biomass (Ameen et al. 2019). For example, Ghasemi et al. (2009a) observed a remarkable reduction in the root biomass of *A. inflatum* seedlings treated with 350-µM Ni. A study on *Oryza sativa* exposed to different Ni-treatment showed the highest Ni accumulation, was associated with the highest reduction in the root FW in the 200-µM Ni-treatment (Rizwan et al. 2018). The same result was reported in *Taraxacum officinale* exposed to Ni stress (Kováčik et al. 2019). Although Ni has been considered as a micronutrient in plants, the amount of Ni in plants is about < 5 mg kg^{-1} of dry weight (DW), when growth on ordinary soils and at least 0.1 mg kg^{-1} DW Ni need to prevent deficiency of Ni in plants (Welch 1995; Chaney et al. 2008). Nevertheless, when the Ni concentration in plants reaches above 50 to 100 mg kg^{-1} (Hassan et al. 2019), plant architecture was demolished, and limited plants to uptake minerals resulted in reduced plant growth (Fashola et al. 2016). One of the reasons for the reduction in plant growth with high Ni concentrations is owing to the lack of other essential elements such as Fe, Cu, and Mn due to their similar chemical properties and competition for uptake through the root (Najafi Kakavand et al. 2019). For example, many transporters involved in Fe absorption and distribution, such as IRON-REGULATED TRANSPORTER 1 (IRT1), can also uptake Ni by roots from the soil and promote Ni accumulation in plants. Additionally, Fe-ligands like nicotianamine involved in Fe translocation from root to shoot can make strong complexes with Ni (Leškova et al. 2020). However, Ni-hyperaccumulator plants can accumulate over 1–3% DW Ni concentration in their tissues (Reeves et al. 2018). For the first time, Ghaderian et al. (2007) reported that *A. inflatum* plants that grow up on the serpentine soils with 1350 µg Ni g^{-1} soils could accumulate more than 3700 µg Ni g^{-1} its shoot DW. In contrast, the current study demonstrated that SA and JA's external use leads to increased Ni concentration in roots and enhanced roots FW of M/NM populations under high Ni-treatment. Similar to our results, some investigations illustrated that JA supplementation moderated Ni-toxicity by enhancing chlorophyll content, CO_2 fixation, and photosynthetic yield, leads to increased plant biomass in *G. max* (Sirhindi et al. 2016) and Cd-stress in *G. max* (Keramat et al. 2009). It was also found that exogenous JA application improved Ni inhibitory effect on mitotic division and reduced the destruction of the root structure leding to improved growth traits in *G. max* (Mir et al. 2018). Likewise, Sirhindi et al. (2015) reported that the external use of JA to Ni toxic soybean plants cause restricted Ni uptake via roots and inhibited the Ni interference with other necessary ionic metals needed for physiological processes improved the biomass of plants. Besides, SA supplementation moderated Ni-toxicity and the amelioration of biomass in *Triticum aestivum* (Siddiqui et al. 2013) and *B. juncea* (Zaid et al. 2019). They suggested that exogenous SA treatment led to increased growth and plant biomass by reducing Ni uptake, improving photosynthetic pigments involved in photosynthesis reaction, increasing nitrogen metabolism and mitotic activities. It is demonstrated that SA reduced abiotic stress, such as heavy metal, through-mediated regulation of main plant-metabolic processes (Khan et al. 2015). Also, SA signaling pathways often cross-talk with other hormone signaling pathways such as JA as a reaction to stress in plants. The interaction between JA and SA signaling pathways can be synergistically or antagonistically, depending on specific stress (Sytar et al. 2019). The external application of SA + JA increased root DW of *Zea mays* under drought stress. (Tayyab et al. 2020). Interestingly, exogenous application SA and JA in Ni-exposure plants probably restrict Ni translocation root-to-shoot by preventing Ni absorption and Ni storage in roots of plants and ameliorating Ni-toxicity effects resulted in biomass accretion (Zaid et al. 2019; Mubarik et al. 2021).

Proline, as a vital osmolyte, has multiple roles in protecting and tolerating plants to abiotic stresses like heavy metals (Petrovic and Krivokapic 2020). Proline is a compatible osmolyte that plays a considerable role in osmotic adjusting, protein stability, membrane integrity, conservation of subcellular structures, and cellular redox-balancing (El-Beltagi et al.

2020). Plants have expended variant protective strategies, including enzymatic/non-enzymatic antioxidant defense systems, to alleviate or obliterate ROS's destructive effects in plant cells. It was suggested that proline has a ROS-scavenging role and elevates antioxidative enzyme activity in plants (Hayat et al. 2012; Kaur and Asthir 2015; Altieri and Nicholls 2020). Rendering to our results, the increase in proline content due to oxidative stress induced by 400-µM Ni in the roots of M/NM *A. inflatum* plants is considerably reduced in plant's roots under SA and JA or SA/JA treatments. However, the proline levels in the plant's roots showed a considerable enhancement compared to control plants. Similarly, research has demonstrated that 100-µM Ni-induced proline accumulation in *Vigna mungo* L. (Gurpreet et al. 2012) and *Sesuvium portulacastrum* L. (Fourati et al. 2020). Also, SA amended the proline contents in *Catharanthus roseus* L. exposed to various Ni doses. However, SA efficaciously decreased Ni-influenced *C. roseus* plants' proline content that grows on different Ni doses medium (Idrees et al. 2013). Likewise, SA and JA's application improved the Ni-tolerance mechanism respectively, in *G. max* (Sirhindi et al. 2016) and *Eleusine coracana* L. (Kotapati et al. 2017) exposed to Ni-stress conditions by increasing proline content. Furthermore, Tayyab et al. (2020) found that external application of combined SA + JA can effectively mitigate drought stress in maize by ameliorating the proline content. The application of SA, JA, and SA + JA triggers proline accumulation, which consecutively elevates the osmotic potential and balances cell redox status, as well as, and improves the antioxidant system function, and finally restrict adverse effects of heavy metals stress in plants (Nazar et al. 2015; Raza et al. 2020). These phiso-biochemical responses are probably in the response of M/NM *A. inflatum* populations to Ni-toxicity, and the signaling cascade is with mediated JA and SA.

Nickel stress considerably alleviates proteins' levels in several plant species, owing to decreased protein synthesis and hydrolyze (Hassan et al. 2019). Decreased protein levels due to heavy metals stress (such as Ni) through various mechanisms, including; (i) high doses of Ni indirectly cause ROS generation, which eventually harms the proteins; (ii) Ni can alter the conformation of proteins by binding functional groups of proteins such as sulfhydryl-groups and consequently blocked of enzymes activity; (iii) as well as Ni-stress, leads to the accumulation of various amino acids like histidine, in the cells of various plant tissues to Ni-detoxify, which resulted in reduced protein synthesis (Dutta et al. 2018; Hassan et al. 2019). The current study showed that the total protein level was reduced in Ni-stressed M *A. inflatum* species over the control plants' roots. However, the supplement of SA, JA, and/or SA + JA with Ni in two *A. inflatum* species showed promotion of the total protein levels in comparison to Ni-treated plants alone. According to previous reports, total protein levels of roots in rice (Rizwan et al. 2018) and wheat (Gajewska et al. 2009) exposed to high doses of Ni showed a 50% reduction than plants without treatments. Alternatively, Sirhindi et al. (2016) found that JA's addition to *G. Max* under Ni-stress resulted in an approximately 60% increment in protein level than Ni-treated plants. Likewise, SA triggered a remarkable improvement of the protein levels in the roots of Cu-stressed *Helianthus annuus* L. (El-Tayeb et al. 2006). It was also explained that the use of exogenous SA + JA in maize reduced the damaging effects of drought stress resulted in an increase in protein levels in comparison to maize plants grown under drought stress without PGR-treatments (Tayyab et al. 2020). A positive effect of exogenously SA, JA, and/or SA + JA treatments on protein contents in roots of M/NM *A. inflatum* populations is probably due to the inhibition of the destructive effect of ROS induced-Ni on the protein structures and their activity.

Heavy metal toxicity induces ROS generation in the root cells by disrupting the electron-transfer-chain in mitochondria and apoplastic space (Farvardin et al. 2020; Hasanuzzaman et al. 2020). Therefore, the over-generation of ROS and consequent oxidative stress in plants can cause great damage to plant cells (Petrov et al. 2015). The high content of Ni indirectly stimulates oxidative stress and enzymatic activity inhibition (Ghori et al. 2019), so that a toxic effect of Ni is related to the ROS regeneration and, as a result, an imbalanced redox state (Georgiadou et al. 2018). Besides, Ni-induced ROS generation induces lipid-peroxidation, protein-oxidation, pigment damage, and harm to DNA (Ameen et al. 2019). On the other hand, accumulated ROS due to Ni-stress can act as a signaling molecule that stimulates phytohormones such as SA and JA, which in turn stimulate the plant's defense responses to Ni-stress, including antioxidant enzymatic system (like SOD, CAT, POD, and APX) and non-enzymatic system (like glutathione and proline) (Sewelam et al. 2016). Besides, antioxidant enzymes are powerful ROS scavengers, so enhancing their activities with increasing Ni concentration is a good

indication of stimulating the antioxidant defense pathway (Giannakoula et al. 2021). The current study showed that with increasing Ni levels, at the same time, H_2O_2 content enhanced, and also SOD, POD, CAT, and APX activities improved in both M/NM populations; however, APX activity did not change in the M population. When plants are subjected to heavy metals, they trigger an antioxidative defense pathway to reduce oxidative stress's negative effects. Interestingly, depending on the genotype and plant species, the kind of stress, and the stress period, the antioxidant defense response varies in different plants (Hasanuzzaman et al. 2020). For example, *Kandelia candel* and *Bruguiera gymnorrhiza* plants were exposed to lead (Pb), Cd, and mercury (Hg) stress, showed an increasing trend in SOD, CAT, and POD activities in roots (Zhang et al. 2007). Also, *O. sativa* exposed to different doses of Ni; increasing the concentration of Ni enhanced the H_2O_2 level in the roots, which subsequently reduces the oxidative stress, improved the enzymatic activity of CAT, POD, and APX, while SOD activity was found to be decreased (Rizwan et al. 2018). Moreover, under Ni-stressed wheat plants, the activity of SOD decreased with increasing H_2O_2 content in roots while CAT and APX and POD activity did not significantly change than control plants (Gajewska and Skłodowska 2008). On the other hand, SA and JA's external use on plants exposed to abiotic stress conditions causes different physio-biochemical responses (Tayyab et al. 2020). According to our results, the external application of SA and JA and/or combined (SA + JA) in roots of both populations of *A. inflatum* treated with 400-μM Ni caused ameliorated oxidative stress by enhancing SOD, CAT, POD activities, which resulted in reduced H_2O_2 levels than roots of plants without treatments. When plants are exposed to abiotic stress, SA and JA are induced as key signaling-molecules in the biochemical response pathways for defense mechanisms in plants. Besides, SA and JA's signaling to fine-tune abiotic stress depends on the character, intensity, and duration of stress exposure (Sewelam et al. 2016). For instance, Yusuf et al. (2012) reported that the ameliorative role of SA on Ni-induced oxidative stress in *B. juncea* and also illustrated that SA supplementation reduced the reverse effects of Ni revealed in the form of the less dose of the abridged generation of H_2O_2. Furthermore, in barley plants exposed to Cd-toxicity, the CAT and APX activities were enhanced, while these enzymes' activity mitigated in the presence of SA. It was also found that the endogenous SA level was incremented to mitigate the damage caused by oxidative stress induced by Cd-toxicity (Metwally 2003). Contrarily, SA elevated the SOD, POD, and CAT activities in *C. roseus* exposed to Ni-stressed (Idrees et al. 2013), *M. arvensis* L. exposed to Cd-treated (Zaid et al. 2020), and watermelon plants under B-toxicity (Moustafa-Farag et al. 2020). Recent molecular researches have proved that SA can regulate genes involved in encoding antioxidative defense mechanisms, and therewith able to ameliorate abiotic stress such as heavy metal (Khan et al. 2015). Moreover, JA is a signaling molecule tightly related to plant defense against biotic and abiotic stresses (Ruan et al. 2019). For instance, in *H. annuus* plants, 50 ml of methyl jasmonate (MeJA) treatment enhanced the ROS content in the root apoplastic space, followed by an increment in the activities of the ROS-scavenging enzymes (Parra-Lobato et al. 2009). Furthermore, Sirhindi et al. (2016) displayed that JA increased the SOD, POD, CAT, and APX activities in *G. max* plants exposed to Ni toxicity. Mir et al. (2018) also reported that JA's supplement has an ameliorating effect on Ni toxicity in *G. max* plants exposed to $NiCl_2$ (4-mM) by improving SOD and CAT activities. So far, there have been several reports on the protective role of JA against metal stress in plants, including *G. max* under Ni toxicity (Sirhindi et al. 2015), *Lycopersicon esculentum* (Bali et al. 2019b), and *Arabidopsis thaliana* (Lei et al. 2020) under Cd stress, *Puccinellia tenuiflora* under B-toxicity toxicity (Zhao et al. 2019), tomato under Pb toxicity (Bali et al. 2019a), and *L. valdiviana* exposed to As stress (Coelho et al. 2020). It is found that JA as a signaling molecule can increase genes expression involved in encoding antioxidative enzymes in plant cells for decreasing heavy metals toxicity by reducing ROS contents. Therefore, JA can protect the macromolecules structure such as proteins as well as cell membranes by reducing the content of H_2O_2 and malondialdehyde in plant cells. (Emamverdian et al. 2020). In the current study, it was determined that the external use of SA + JA resulted in a significantly reduced H_2O_2 content by enhancing antioxidative enzyme activity in the roots of both M/NM populations. However, the high activity of the APX enzyme in the M-population's root was observed only along whit the lower doses of SA and JA. The same results were obtained by Tayyab et al. (2020) on *Z. mays* plants under drought stress. Notably, SA and JA play a substantial role in triggering the expression of genes associated with defense mechanisms by causing oxidation to alter the signaling pathway constituents (Fobert and Després 2005; Kalaivani et al. 2016; Raza et al. 2020, 2021). These investigations illustrated that external SA and JA application could efficiently mitigate heavy metals toxicity (e.g., Ni) in plants.

Additionally, PCA plot results show that two populations of *A. inflatum* display similar trends in physiological and biochemical reactions in response to SA, JA, and SA + JA treatment under Ni-toxicity conditions. These results propose that SA and JA, especially SA + JA, under Ni-toxicity is more efficient in enhancing antioxidant enzymatic capacity and proline content to decrease oxidative stress induced by Ni-toxicity. Although there are many investigations on SA and/or JA's positive role to restrict toxic effect-induced by heavy metals such as Ni in plants; however, no study has been reported on the beneficial effects of SA + JA combination on heavy metal toxicity. Hence, this study serves as the main purpose to evaluate the influential role of SA + JA combination in enhancing Ni-tolerance in M/NM *A. inflatum* populations.

Conclusion

Although Ni is considered a micronutrient for plants, high concentrations of Ni can lead to metabolic disorders, ionic imbalance, and oxidative stress in the plant and ultimately inhibit plant growth and development. Nevertheless, supplementation of SA and JA in the present study, shown ameliorating effects and play an essential role in overcoming Ni-toxicity. Our findings demonstrated that exogenous use of JA and SA, especially combined of SA + JA, in both M/NM *A. inflatum* populations had substantial effects on improving the root biomass, antioxidant defense responses, and proline contents (as an osmolyte), which in turn mitigate the oxidative stress due to Ni-toxicity (Fig. 7). In conclusion, the SA, JA, and/or SA + JA supplementation can partially exclude the damaging effects of Ni-induced oxidative stress in two M/NM populations of *A. inflatum* by adjusting defense signaling pathways and antioxidant mechanisms. However, the mechanisms of interaction between heavy metals and hormones affecting abiotic stresses such as SA and JA is not yet crystal clear and needs further investigation.

Abbreviations

APX**,** Ascorbate peroxidase; CAT, Catalase; FW, Fresh weight; JA**,** Jasmonic acid; M, Metallicolous; Ni, Nickel; NM**,** Non-metallicolous; POD**,** Peroxidase; PGR, Plant growth regulator; ROS, Reactive oxygen species; SA, Salicylic acid; SOD, Superoxide dismutase

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SNK performed the experiment, writing, editing, statistical analysis, figure construction, and finalize the manuscript; NK supervised the research; HRG review and editing; AR reviewed, edited, writing, figure construction, and finalize the

manuscript; MC performed the statistical analysis, figure construction, reviewed, and writing;, MM supervised the research, review, and writing.

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Figures

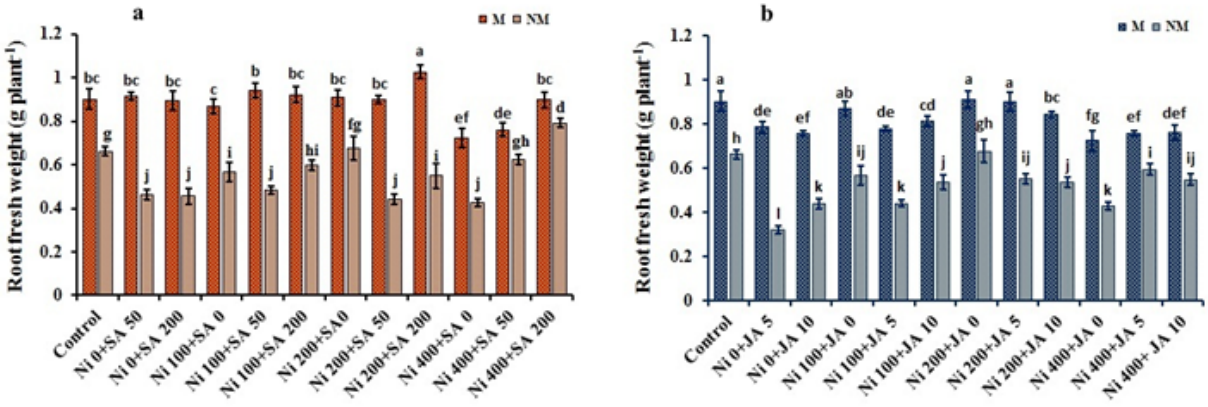


Figure 1

Influence of (a) SA (0, 50, 200 µM), and (b) JA (0, 5, 10 µM) on roots FW of M/NM populations of A.inflatum exposed to Ni (0, 100, 200, 400 µM) conditions. Data are introduced as a mean of each treatment group ± SE (n=3). Different letters presented statistical differences in means by the Duncan test (at $p \leq 0.05$).

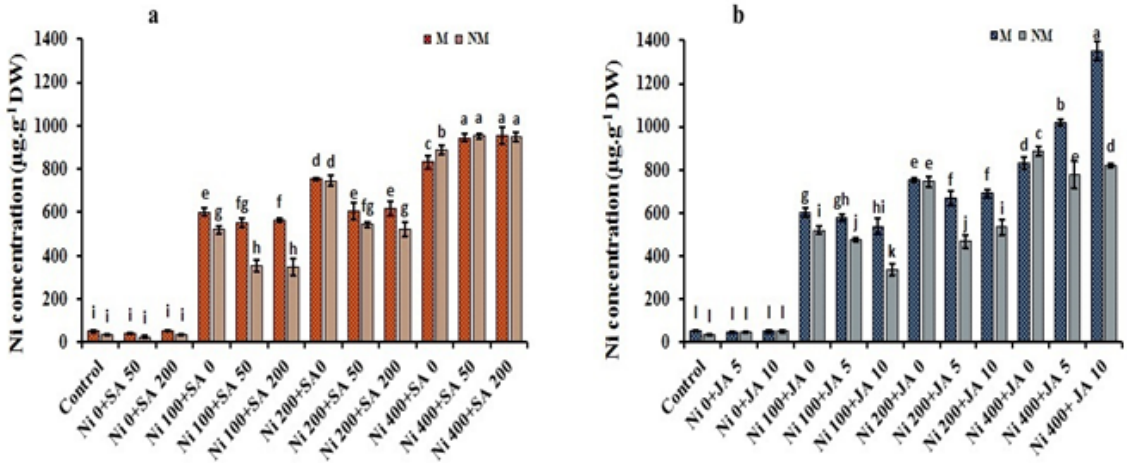


Figure 2

Influence of (a) SA (0, 50, 200 µM), and (b) JA (0, 5, 10 µM) on Ni accumulation in roots of M/NM populations of A.inflatum exposed to Ni (0, 100, 200, 400 µM) conditions. Data are introduced as a mean of each treatment group ± SE (n=3). Different letters presented statistical differences in means by the Duncan test (at $p \leq 0.05$).

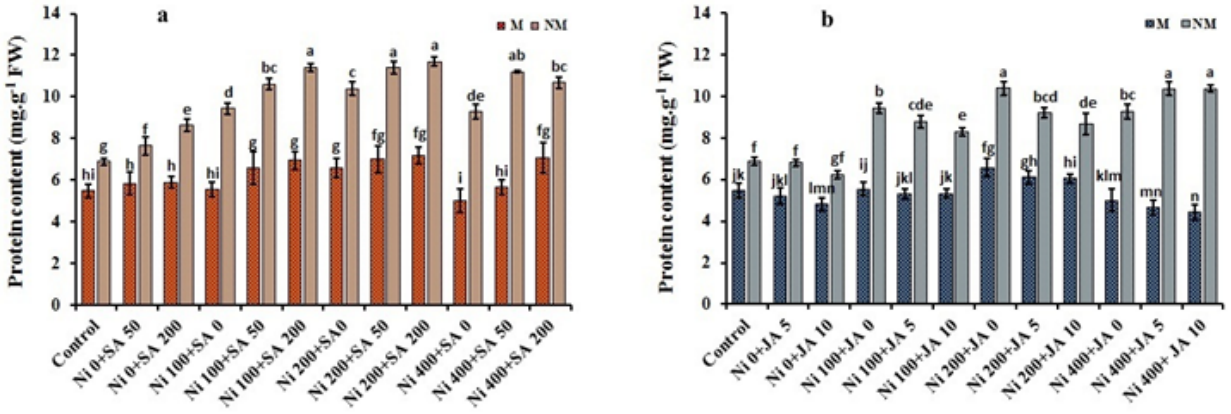


Figure 3

Influence of (a) SA (0, 50, 200 µM), and (b) JA (0, 5, 10 µM) on protein levels in roots of M/NM populations of A.inflatum exposed to Ni (0, 100, 200, 400 µM) conditions. Data are introduced as a mean of each treatment group ± SE (n=3). Different letters presented statistical differences in means by the Duncan test (at $p \leq 0.05$).

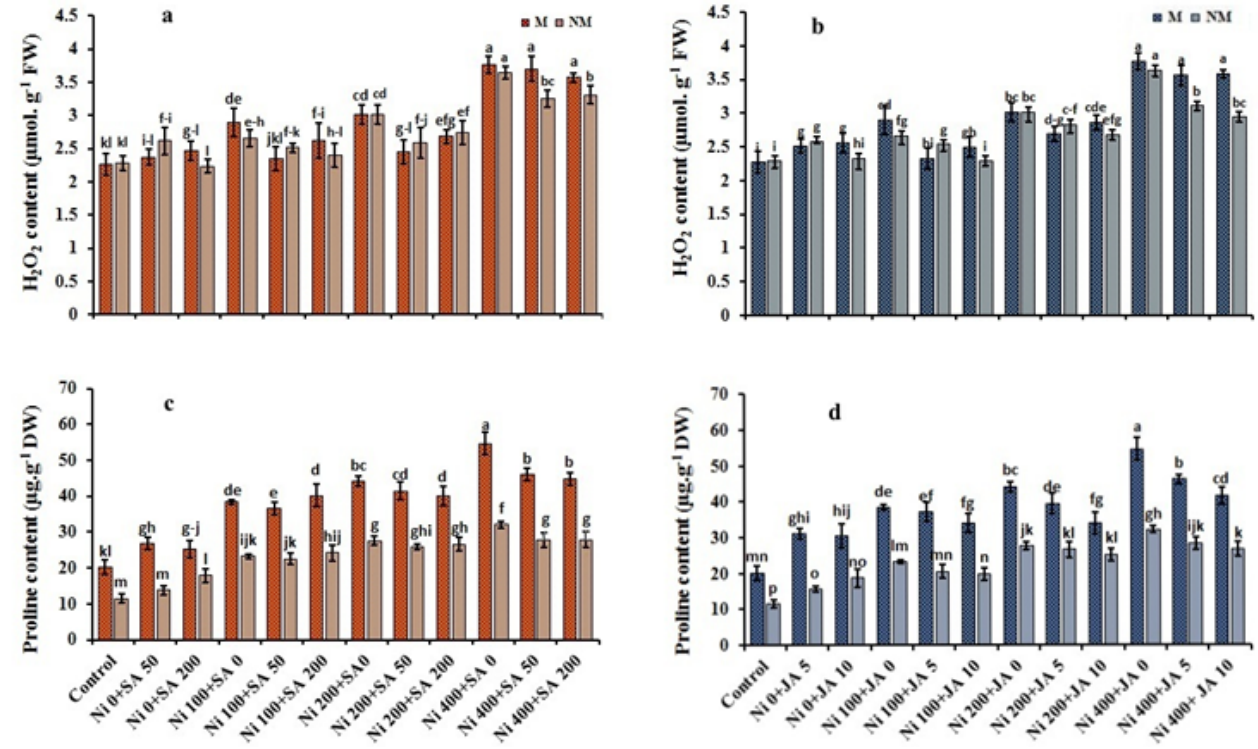


Figure 4

Influence of SA (0, 50, 200 µM), and (b) JA (0, 5, 10 µM) on (a, b) H2O2 levels and (c, d) proline contents in roots of M/NM populations of A.inflatum exposed to Ni (0, 100, 200, 400 µM) conditions. Data are introduced as a mean of each treatment group ± SE (n=3). Different letters presented statistical differences in means by the Duncan test (at $p \leq 0.05$).

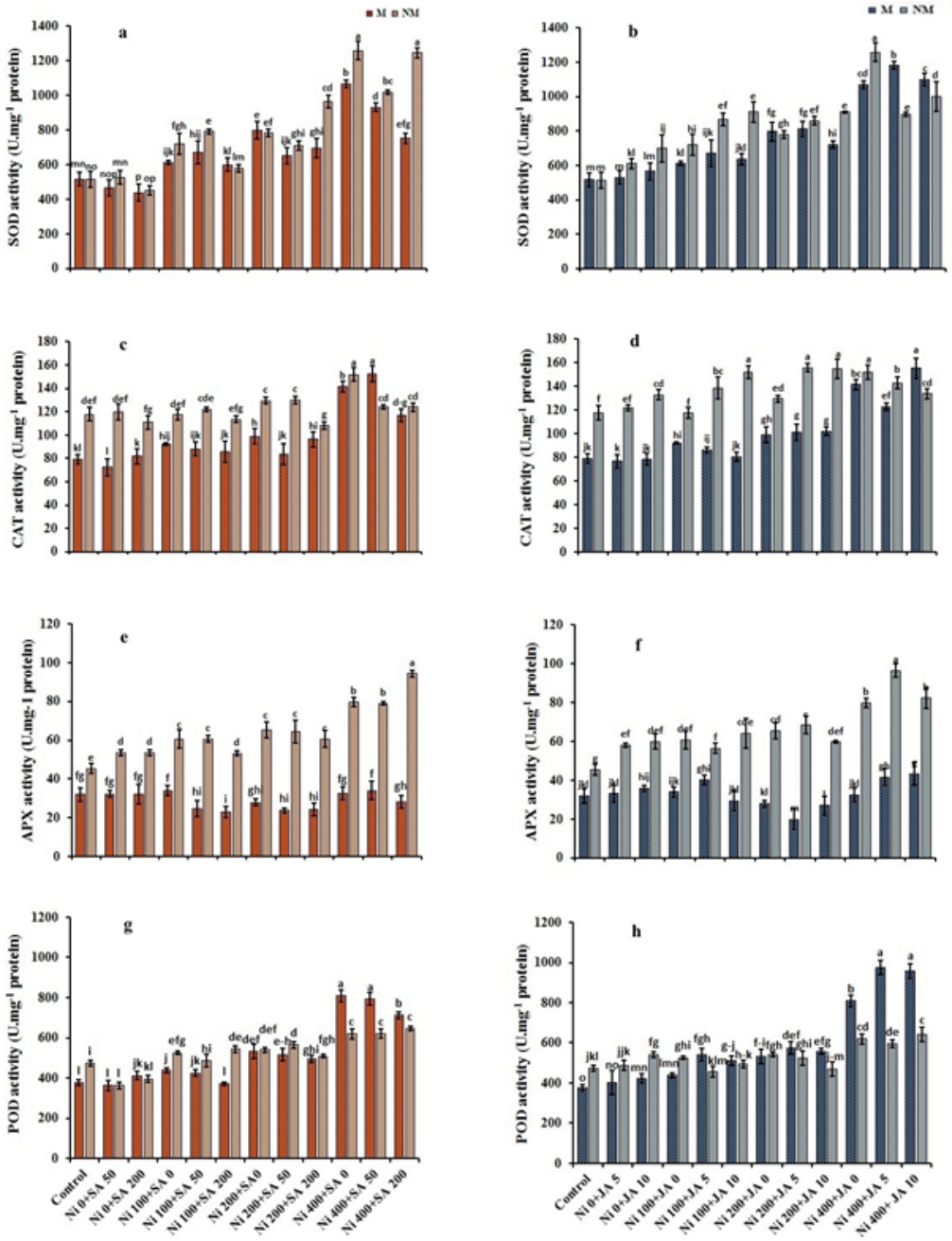


Figure 5

Influence of SA (0, 50, 200 μM), and (b) JA (0, 5, 10 μM) on activities of (a, b) SOD, (c, d) CAT, (e, f) APX, and (g, h) POD in roots of M/NM populations of A.inflatum exposed to Ni (0, 100, 200, 400 μM) conditions. Data are introduced as a mean of each treatment group ± SE (n=3). Different letters presented statistical differences in means by the Duncan test (at $p \leq 0.05$).

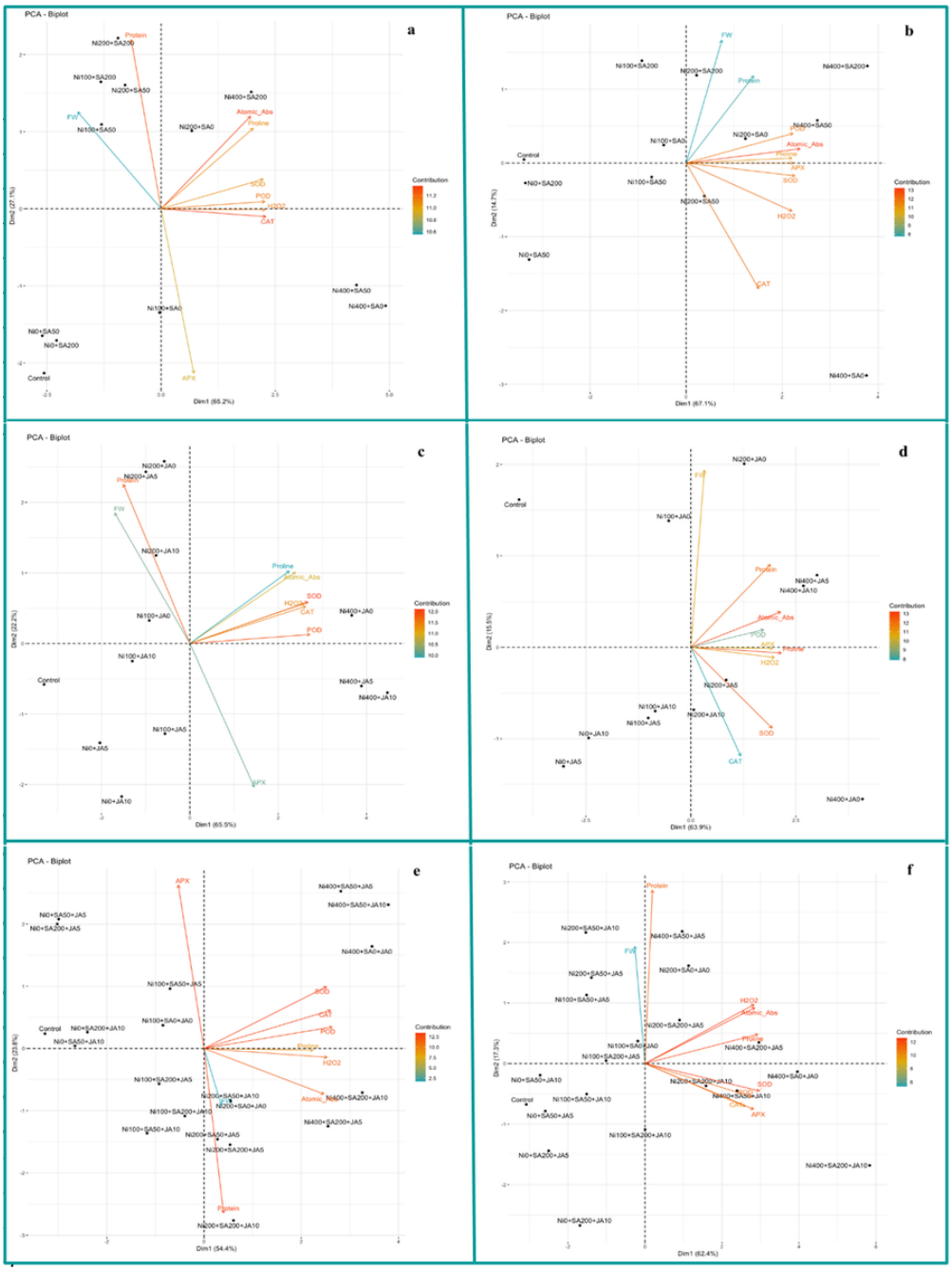


Figure 6

Principal component analysis (PCA) of Ni-stress indexes such as FW, Ni contents, proline, and antioxidative enzymes in roots of (a, c, e) M population and (b, d, f) NM population of A. inflatum treated with SA, JA, and SA+JA combination under Ni-toxicity, respectively.

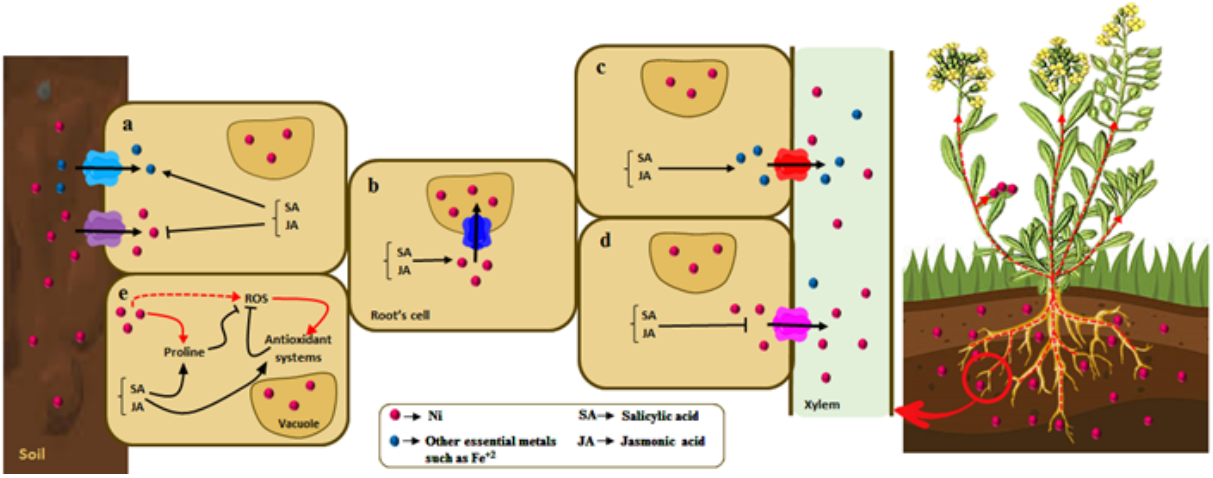


Figure 7

Schematic illustration of the influence of SA, JA, and/or SA+JA supplementation on roots of A. inflatum exposed to Ni stress. SA, JA, and SA+JA supplementation moderated Ni-toxicity and as well as improved plant biomass in plants by (a) decrease Ni uptake by roots and increase other essential metals (such as Fe); (b) Ni accumulation in roots cells; (c) and increase translocation of essential metals to shoot, (d) and restrict Ni translocation from root to shoot. Besides, these PGRs supplementation increased plant tolerance to Ni-toxicity by (e) ameliorate the antioxidant system and increase proline level in plant roots.