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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Received: 10 July 2021 / Accepted: 21 March 2022 / Published online: 11 April 2022
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Abstract
Phytohormones, including salicylic acid (SA) and jasmonic acid (JA) 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 different Ni concentrations (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 concentration (400 μM) reduced roots fresh weight in both populations than in control. However, external application of individual SA and JA or combined SA + JA in higher doses had ameliorated roots biomass by mitigating Ni-toxicity, especially in the NM population, in comparison to 400 μM Ni. 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 by improving the activity of superoxide dismutase, peroxidase, catalase, and ascorbate peroxidase 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 Ni translocation to the shoot, accumulating in roots, and enhancing antioxidant defense systems.

Keywords Alyssum inflatum · Antioxidant defense · Ni-toxicity · Oxidative stress · Phytohormones

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

Handling Editor: Pramod kumar nagar.

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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; Raza et al. 2022). 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; Lešková 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 causes 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 (Brassica juncea L.) (Abd_Allah et al. 2019; Khan et al. 2020), and tomato (Solanum lycopersicum L.) (Jahan et al. 2020) leads to reduced growth-related traits by inhibiting photosynthesis, 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.

Likewise, when plants are exposed to abiotic stresses such as heavy metals, 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; Sabagh et al. 2021). Jasmonic acid (JA) and salicylic acid (SA) arbitrated signaling pathways have been shown as potential tools in increasing plant tolerance against stress conditions (Caarls et al. 2015). Jasmnates [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, 2021a). Various studies have 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 soybean (Glycine max L.) (Keramat et al. 2009) and menthol mint (Mentha arvensis L.) (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 a stress messenger involved in abiotic stress signaling, a plant defense that responds to heavy metal toxicity (Zaid et al. 2019; Rhaman et al. 2020). Numerous studies show SA has a role in decreasing 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 decreasing hydrogen peroxide (H\(_2\)O\(_2\)) contents. Exogenously applied SA remarkably improved antioxidative enzymes' activities in duckweed (Lemma minor L.) under cadmium (Cd) toxicity (Lu et al. 2018) and water lily (Nymphaea tetragona) (Gu et al. 2018), sorghum (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; Mubarak et al. 2021; Sabagh et al. 2021). It is well-identified that an antagonistic effect between the interaction of SA and JA signaling pathways in various plants against biotic stress (Li et al. 2019). However, it has been reported that the cross-talk synergistic effect between SA and JA signaling pathways caused modulation in expression of OsHPL3 gene as an intermediate compound of the oxylipin pathway and increased resistance 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 the interactive effects of SA/JA in plants exposed to heavy metal toxicity (Najafi-Kakavand et al. 2019).

The species of Alyssum inflatum Nyár. belongs to the Brassicaceae family, and it is native to serpentine and non-serpentine soils of the west of Iran (Ghaderian et al. 2007). Besides, A. inflatum is considered a Ni-hyperaccumulator plant (can accumulate Ni > 1000 µg 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 antioxidative enzyme activities of roots of two metallocolous (M) and non-metallocolous (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, 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, eight seeds of A. inflatum were planted in a pot [(450 ml) filled with perlite: sand (2:1 mixture)] for each replicate, individually. Then each pot was placed in a bucket. The pots were irrigated daily with 300 ml tap water inside each bucket containing a pot till germination and subsequently with Hoagland’s solution as described by Najafi-kakavand et al. (2019). All A. inflatum seedlings were grown in a growth chamber under controlled conditions (20–25 °C, 16/8 h light/dark cycle, PPFD of 140 μmol 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). Once per week, treatments were performed. In this experiment, to prevent possible interference and antagonistic effect on the absorption between SA and JA in SA + JA combination treatments, SA and Ni were used as a mixture simultaneously with Hoagland solution and JA as a foliar spray (Najafi-kakavand et al. 2019). 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 °C for further examination. To obtain dry matter, root tissue was exposed to full dehydration in an oven at 70 °C for 3 days.

Nickel Contents Evaluation

Nickel concentration was measured according to Ghasemi et al. (2009a, b). Briefly, Ni concentrations in 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 H₂O₂ (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 H₂O₂ Concentration

Hydrogen peroxide (H₂O₂) contents were measured according to the Sergiev et al. (2000) method. Briefly, H₂O₂ was extracted from 50 mg fresh root materials by homogenization with 5 ml of cold trichloro-acetic acid (TCA) [0.1% (W/V)]. The homogenates were centrifuged at 12,000×g (15 min, 4 °C). To measure the H₂O₂ content, the supernatant (0.5 ml) was mixed with 0.5 ml phosphate buffer (200 mM; pH 7.0) and 1 ml potassium-iodide (1 M). Eventually, the H₂O₂ concentration was assayed according to the absorbance of the mixed solution at 390 nm by using a standard curve and expressed as μmol g⁻¹ of root FW.

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 1 mM ethylene-diamine-tetraacetic acid (EDTA) and polyvinylpyrrolidone (PVP) [% 1(W/V)], and afterwards centrifuged (13,000×g; 15 min; 4 °C). The supernatant 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 root extraction, 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 assay solution included 800 μl of phosphate buffer (50 mM; pH 7.0), 100 μl of 30 mM H₂O₂, and 100 μl of root extraction. The disintegration of H₂O₂ was monitored by a decrease in absorbance at 240 nm. The enzyme activity was stated as U mg⁻¹ protein.

The peroxidase (POD) activity was examined following the method of Chance and Maehly (1955). The reaction solution consisted of 100 μl root extraction, phosphate buffer (200 mM; pH 7), guaiacol (1%), and 1 mM H₂O₂. 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 root extraction (50 μL), phosphate potassium buffer (50 mM; pH 7), 1 mM EDTA, 5 mM ascorbate, and 1 mM H₂O₂. 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 (4000 g; 10 min). Then, 0.5 ml of
supernatant, 0.5 ml glacial acetic acid and 1 ml ninhydrin reagent were added and held in a water bath (100 °C) for 1 h. Finally, the reaction was terminated by placing the sample tubes into a cold bath. The product was extracted by combining 1 ml of toluene with the reaction solution by vortexing for 1 min. Finally, the supernatant’s absorbance is recorded at 520 nm. Proline concentrations were calculated using a standard L-proline curve [the calibration curve was previously generated using 0, 5, 10, 15, 20, 25, 30, and 35 μM L-proline], and expressed as μg g⁻¹ of root DW.

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 ≤ 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 analysis included the three-way effects of Ni, SA, and JA. In this group, SA0 and JA0 were also used in all Ni levels to analyze variance with 20 treatments. The data expressed 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 a middle dose of Ni (200 μM) treatment, root fresh weight (FW) of both M and NM plants did not indicate significant differences with control plants (p ≤ 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 by 19.4% and 46.1% 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. According to Table 1, in the presence of high concentrations of SA and JA (200 μM SA + 10 μM JA), a reduction in FW (18.7%) was appeared in the NM population compared to the control, while in the same treatment, a 16.3% increase in FW in the M population was observed. Application of 200 μM SA + 10 μM JA on M population increased the roots FW by 11.7% under 400 μM Ni stress than control plants. Conversely, 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.

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 concentration in control plants. Similarly, JA supplementation also enhanced Ni accumulation to 200 μM Ni stress over control treatments. According to Table 1, supplementation of SA0 + JA0 on the NM population resulted in 26.7% more Ni accumulation than control plants. However, supplementation of SA0 + JA0 on M population decreased Ni accumulation by 16.3% in comparison to control plants.
Table 1 Influence of combined SA + JA on Ni contents, FW, protein levels, H$_2$O$_2$ levels, and proline contents in roots of M and 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$_2$O$_2$ content (µmol g$^{-1}$ FW) | Proline content (µg g$^{-1}$ DW) |
|------------|----------------------------------|---------------------|-------------------------------|--------------------------------------|-----------------------------|
| M          | NM                               | M                   | M                             | M                                    | M                           |
| Control    | 50.9 ± 4.03$^{a}$                | 34.5 ± 3.74$^{a}$   | 0.90 ± 0.04$^{a}$             | 0.66 ± 0.02$^{a}$                   | 5.47 ± 0.34$^{a}$            |
| (SA50 + JA5) µM | 46.5 ± 4.14$^{a}$                | 46.4 ± 4.03$^{a}$   | 0.68 ± 0.02$^{a}$             | 0.54 ± 0.02$^{a}$                   | 4.77 ± 0.24$^{a}$            |
| (SA50 + JA10) µM | 51.8 ± 3.81$^{a}$                | 40.0 ± 5.56$^{a}$   | 0.67 ± 0.03$^{a}$             | 0.62 ± 0.02$^{a}$                   | 5.68 ± 0.46$^{a}$            |
| (SA200 + JA5) µM | 51.9 ± 3.89$^{a}$                | 40.0 ± 4.58$^{a}$   | 0.81 ± 0.02$^{b}$             | 0.53 ± 0.04$^{a}$                   | 4.56 ± 1.10$^{a}$            |
| (SA200 + JA10) µM | 52.8 ± 3.18$^{a}$                | 47.1 ± 4.36$^{a}$   | 1.07 ± 0.01$^{b}$             | 0.38 ± 0.05$^{a}$                   | 5.41 ± 0.64$^{a}$            |
| Ni100 µM   | 602 ± 18.4$^{b}$                 | 520 ± 19.3$^{b}$    | 0.86 ± 0.03$^{b}$             | 0.56 ± 0.04$^{b}$                   | 5.54 ± 0.34$^{b}$            |
| (Ni100 + SA50 + JA5) µM | 797 ± 9.77$^{b}$                | 242 ± 16.0$^{b}$    | 0.72 ± 0.01$^{b}$             | 0.65 ± 0.03$^{b}$                   | 5.23 ± 0.64$^{b}$            |
| (Ni100 + SA50 + JA10) µM | 838 ± 19.7$^{b}$                | 254 ± 25.5$^{b}$    | 0.76 ± 0.01$^{b}$             | 0.32 ± 0.01$^{b}$                   | 6.58 ± 0.66$^{b}$            |
| (Ni100 + SA200 + JA5) µM | 877 ± 28.8$^{b}$                | 259 ± 24.0$^{b}$    | 0.97 ± 0.01$^{de}$            | 0.63 ± 0.01$^{b}$                   | 5.74 ± 0.33$^{b}$            |
| (Ni100 + SA200 + JA10) µM | 895 ± 61.6$^{b}$                | 273 ± 23.0$^{b}$    | 1.16 ± 0.02$^{b}$             | 0.49 ± 0.04$^{b}$                   | 6.09 ± 0.49$^{b}$            |
| Ni200 µM   | 753 ± 8.05$^{b}$                 | 745 ± 24.5$^{b}$    | 0.91 ± 0.03$^{b}$             | 0.67 ± 0.05$^{b}$                   | 6.58 ± 0.45$^{b}$            |
| (Ni200 + SA50 + JA5) µM | 963 ± 24.9$^{b}$                | 288 ± 69.0$^{b}$    | 0.73 ± 0.03$^{b}$             | 0.49 ± 0.01$^{b}$                   | 6.96 ± 0.38$^{b}$            |
| (Ni200 + SA50 + JA10) µM | 976 ± 37.4$^{b}$                | 357 ± 12.5$^{b}$    | 0.77 ± 0.02$^{b}$             | 0.67 ± 0.03$^{b}$                   | 6.29 ± 0.36$^{b}$            |
| (Ni200 + SA200 + JA5) µM | 1034 ± 12.7$^{b}$               | 322 ± 22.5$^{b}$    | 0.79 ± 0.01$^{b}$             | 0.59 ± 0.02$^{b}$                   | 6.88 ± 0.50$^{b}$            |
| (Ni200 + SA200 + JA10) µM | 986 ± 18.2$^{e}$                | 344 ± 27.0$^{e}$    | 0.93 ± 0.02$^{e}$             | 0.47 ± 0.02$^{e}$                   | 8.21 ± 0.26$^{e}$            |
| Ni400 µM   | 831 ± 29.1$^{b}$                 | 886 ± 19.5$^{f}$    | 0.72 ± 0.04$^{km}$            | 0.42 ± 0.02$^{a}$                   | 5.01 ± 0.55$^{pq}$           |
| (Ni400 + SA50 + JA5) µM | 1268 ± 16.2$^{c}$               | 743 ± 9.50$^{c}$    | 0.74 ± 0.03$^{k}$             | 0.71 ± 0.03$^{k−n}$                 | 5.07 ± 0.46$^{pq}$           |
| (Ni400 + SA50 + JA10) µM | 1310 ± 39.8$^{c}$               | 818 ± 37.5$^{c}$    | 0.93 ± 0.02$^{f}$             | 0.53 ± 0.02$^{e}$                   | 4.55 ± 0.34$^{f}$            |
| (Ni400 + SA200 + JA5) µM | 1151 ± 37.3$^{c}$               | 803 ± 25.0$^{b}$    | 0.99 ± 0.01$^{cd}$            | 0.57 ± 0.04$^{b}$                   | 6.70 ± 0.35$^{c}$            |
| (Ni400 + SA200 + JA10) µM | 1345 ± 16.9$^{c}$               | 900 ± 21.0$^{f}$    | 1.02 ± 0.01$^{c}$             | 0.55 ± 0.03$^{a}$                   | 6.17 ± 0.30$^{a}$            |

Abbreviations are defined in the main text.

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 $p \leq 0.05$).
Ni-treated plants. Likewise, increasing JA concentration upsurgs 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 (50 and 200 µM) application, protein contents increased under 400 µM Ni stress in the roots of NM population compared to 400 µM Ni treatment. However, a significant increase in protein content was observed in the M population root only in 400 µM Ni + 200 µM SA treatments concerning plants treated by 400 µM Ni. The same results were observed in 400 µM Ni + 5 µM JA and 400 µM Ni + 10 µM JA treatments in NM population 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 and NM plants simultaneously increased the protein content, especially under 200 μM Ni stress compared to control. 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 and NM plants demonstrated a significant accumulation of \( \text{H}_2\text{O}_2 \), which was not observed in control plants (Fig. 4a,b). Whereas, the external application of SA and/or JA in plants exposed to Ni induced a declining trend in \( \text{H}_2\text{O}_2 \) content in the roots of both populations, especially NM species, compared to 400 μM Ni-treatment. Additionally, the simultaneous application of SA + JA in plants exposed to Ni showed a decreasing trend in \( \text{H}_2\text{O}_2 \) levels (Table 1). For instance, in 400 μM Ni + 50 μM SA + 10 μM JA treatment, the \( \text{H}_2\text{O}_2 \) content decreased by 16.6% and 19.6% in M and NM plants, respectively, compared with plants treated with 400 μM Ni.

**The Activity of Antioxidant Enzymes**

High doses of Ni indirectly lead to the generation of \( \text{H}_2\text{O}_2 \), which in turn stimulates the activity of antioxidative enzymes (SOD, CAT, POD, and APX) under Ni stress in M and 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. 5 e,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 compared to the control plants. The activities of four antioxidative enzymes in the roots of M and NM populations exposed to different Ni concentrations were changed in response to SA and JA alone and the simultaneous application of SA + JA. The percent of SOD, POD, and

![Fig. 4](image-url) Influence of SA (0, 50, and 200 μM) (red graphs), and JA (0, 5, and 10 μM) (blue graphs) on (a) and (b) \( \text{H}_2\text{O}_2 \) levels and (c) and (d) proline contents in roots of M and NM populations of *A. inflatum* exposed to Ni (0, 100, 200, and 400 μM) conditions. Data are presented as a mean of each treatment group ± SE (n=3). Different letters presented statistical differences in means by the Duncan test (at p ≤ 0.05).
Fig. 5 Influence of SA (0, 50, and 200 μM) (red graphs), and JA (0, 5, and 10 μM) (blue graphs) on activities of (a, b) SOD, (c, d) CAT, (e, f) APX, and (g, h) POD in roots of M and NM populations of A. inflatum exposed to Ni (0, 100, 200, and 400 μM) conditions. Data are presented as a mean of each treatment group ± SE (n = 3). Different letters presented statistical differences in means by the Duncan test (at p ≤ 0.05).
CAT activities was remarkably improved with the application of SA and/or JA than the control plants in both populations. However, the external use of SA in M-population exposed to low doses of Ni (100 and 200 μM) was led to the reduced enzymatic activity of APX compared to control. Similarly, these results were observed in the application of SA + JA simultaneously with Ni in low doses in APX activity in the M-population. Also, with an increasing concentration of SA and JA (200 μM SA + 10 μM JA), POD activity in the M population was increased by 20.7% in comparison to untreated plants. On the contrary, POD activity was reduced at 50 μM SA + 10 μM JA and 200 μM SA + 5 μM JA treatments in the NM population compared to the control. According to Table 2, the presence of SA + JA at low concentrations (50 μM SA + 5 μM JA) resulted in a reduction in SOD activity in the NM population compared to control, while a higher concentration of SA (200 μM SA + 5 μM JA) led to increased activity of this enzyme. Likewise, the enzymatic activity of SOD under 200 μM SA + 50 μM JA treatment was increased by 21.4% in the M population with respect to control. The highest enzyme activity, i.e., 13.5% (SOD), 23.8% (CAT), 7.93% (APX), and 18.1% (POD) were recorded in the roots of M plants by 400 μM Ni + 50 μM SA + 10 μM JA treatment compared to plants under high dose of Ni (400 μM).

### Table 2

| Treatments             | SOD activity (U mg⁻¹ protein) | APX activity (U mg⁻¹ protein) | POD activity (U mg⁻¹ protein) | CAT activity (U mg⁻¹ protein) |
|------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
|                        | M                | NM               | M                | NM               | M                | NM               | M                | NM               |
| Control                | 516 ± 41.1       | 513 ± 45.8       | 31.9 ± 3.57     | 45.3 ± 2.72     | 376 ± 13.8       | 473 ± 15.0       | 79.1 ± 3.84     | 117 ± 6.01       |
| (SA50 + JA5) μM        | 664 ± 17.6       | 399 ± 34.9       | 38.8 ± 2.68     | 52.8 ± 1.11     | 415 ± 30.8       | 500 ± 31.5       | 84.8 ± 8.51     | 113 ± 4.74       |
| (SA50 + JA10) μM       | 504 ± 60.8       | 495 ± 30.0       | 28.3 ± 2.99     | 49.0 ± 4.62     | 442 ± 27.4       | 408 ± 36.4       | 78.4 ± 11.2     | 130 ± 13.4       |
| (SA200 + JA5) μM       | 569 ± 63.9       | 747 ± 102        | 39.7 ± 1.91     | 55.6 ± 2.45     | 441 ± 52.0       | 395 ± 27.1       | 84.1 ± 4.31     | 114 ± 9.98       |
| (SA200 + JA10) μM      | 627 ± 21.1       | 683 ± 81.2       | 33.1 ± 3.56     | 64.9 ± 0.69     | 454 ± 39.3       | 474 ± 21.6       | 79.3 ± 6.92     | 121 ± 28.50      |
| Ni100 μM               | 609 ± 12.4       | 719 ± 60.4       | 33.9 ± 2.52     | 60.6 ± 4.65     | 438 ± 13.5       | 526 ± 8.32       | 92.2 ± 0.85     | 117 ± 4.77       |
| (Ni100 + SA50 + JA5) μM | 725 ± 25.8       | 572 ± 39.6       | 33.5 ± 2.75     | 52.8 ± 2.26     | 557 ± 67.0       | 473 ± 21.0       | 99.4 ± 13.8     | 127 ± 7.82       |
| (Ni100 + SA50 + JA10) μM | 562 ± 67.7       | 738 ± 49.8       | 23.9 ± 1.07     | 51.7 ± 4.03     | 475 ± 36.3       | 417 ± 23.9       | 96.6 ± 904      | 123 ± 20.8       |
| (Ni100 + SA200 + JA5) μM | 594 ± 23.9       | 896 ± 30.1       | 28.2 ± 3.84     | 61.4 ± 4.41     | 572 ± 42.9       | 400 ± 13.5       | 95.9 ± 5.26     | 113 ± 4.03       |
| (Ni100 + SA200 + JA10) μM | 702 ± 32.0       | 979 ± 23.5       | 27.6 ± 2.79     | 65.8 ± 2.60     | 569 ± 66.7       | 496 ± 30.6       | 97.6 ± 4.85     | 112 ± 6.70       |
| Ni200 μM               | 797 ± 53.2       | 780 ± 21.8       | 27.9 ± 1.77     | 65.3 ± 4.12     | 532 ± 35.1       | 540 ± 12.1       | 99.0 ± 4.65     | 129 ± 2.90       |
| (Ni200 + SA50 + JA5) μM | 728 ± 66.0       | 713 ± 82.4       | 23.0 ± 1.63     | 46.5 ± 0.81     | 587 ± 35.0       | 440 ± 23.9       | 108 ± 15.1      | 109 ± 10.8       |
| (Ni200 + SA50 + JA10) μM | 812 ± 47.2       | 730 ± 91.1       | 23.5 ± 1.37     | 54.8 ± 3.03     | 585 ± 52.4       | 357 ± 13.5       | 121 ± 8.14      | 109 ± 15.4       |
| (Ni200 + SA200 + JA5) μM | 784 ± 50.6       | 893 ± 35.9       | 21.8 ± 1.61     | 64.8 ± 2.96     | 573 ± 30.5       | 556 ± 23.0       | 114 ± 1.62      | 155 ± 4.76       |
| (Ni200 + SA200 + JA10) μM | 712 ± 62.9       | 1169 ± 31.3      | 21.9 ± 1.81     | 62.4 ± 4.50     | 616 ± 20.5       | 531 ± 25.4       | 111 ± 6.87      | 191 ± 7.87       |
| Ni400 μM               | 1067 ± 21.6      | 1257 ± 52.7      | 32.5 ± 3.46     | 79.7 ± 2.38     | 809 ± 29.7       | 619 ± 26.1       | 141 ± 4.37      | 151 ± 5.85       |
| (Ni400 + SA50 + JA5) μM | 1154 ± 54.8      | 953 ± 110         | 40.4 ± 5.02    | 65.2 ± 3.14     | 825 ± 31.7       | 537 ± 32.0       | 160 ± 7.99      | 127 ± 6.49       |
| (Ni400 + SA50 + JA10) μM | 1233 ± 69.2      | 1268 ± 93.1      | 35.3 ± 1.41     | 73.5 ± 5.00     | 987 ± 68.6       | 619 ± 32.7       | 185 ± 4.41      | 161 ± 7.95       |
| (Ni400 + SA200 + JA5) μM | 863 ± 20.4       | 1241 ± 40.7      | 24.3 ± 1.48     | 62.4 ± 5.45     | 881 ± 39.5       | 570 ± 41.2       | 129 ± 3.19      | 202 ± 5.15       |
| (Ni400 + SA200 + JA10) μM | 911 ± 42.1       | 1657 ± 43.2      | 25.1 ± 1.54     | 88.7 ± 4.97     | 910 ± 57.7       | 737 ± 45.1       | 156 ± 6.77      | 255 ± 6.24       |

Abbreviations are defined in the main text.

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 ≤ 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-dependent manner. The proline levels in the roots of both M and 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 and 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 compared to control plants. 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 + 10 μM JA in the root of NM species, respectively, compared to the root of control plants (Table 1). However, SA + JA treatments simultaneously in plants of M and NM populations exposed to Ni-toxicity caused a significant decrease in proline content compared to plants exposed to Ni stress alone.

**Principal Component Analysis**

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, H₂O₂, and Ni concentration were positively correlated. In contrast, FW and protein slightly correlated 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, H₂O₂, 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 combining SA + JA treatments, which was associated with the highest enzymatic activity, proline accumulation, and H₂O₂.

**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 and NM populations of *A. inflatum* under different Ni concentrations. High Ni accumulation in various plant tissues has toxic effects on various growth and physiological processes, such as inhibiting growth and photosynthetic capability, leading to decreased 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 consequently, lead to a reduction in roots FW. Similar results were observed in the FW of shoots of both M and NM populations of *A. inflatum* (Najafi-kakavand et al. 2019). Besides, it was found that the roots of plants suffer the most damage compared to the shoots, where the roots are exposed to a huge quantity 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 rice 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 exceeds 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 Ni over 1% to 3% in shoot DW (Reeves et al. 2018). For the first time,

Fig. 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.
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 Ni more than 3700 μg g⁻¹ in its shoot DW. As shown in our previous report, an approximate value of 1390 μg g⁻¹ Ni in their shoots DW were accumulated in both M and NM populations of A. inflatum under 400 μM (Najafi-kakavand et al. 2019), which were about 1.7- and 1.6-times higher than the Ni quantities in roots of M and NM plants, respectively. This indicates that A. inflatum is a Ni-hyperaccumulator plant. In contrast, the current study demonstrated that SA and JA’s external use increases Ni concentration in roots and enhanced roots FW of M and NM populations under high Ni-treatment. According to our previous study, treatment with SA and JA in both populations of A. inflatum led to a notable reduction in Ni accumulation in the shoots of these plants (Najafi-kakavand et al. 2019).

Similar to our results, some investigations illustrated that JA supplementation moderated Ni-toxicity by enhancing chlorophyll content, CO₂ fixation, and photosynthetic yield, leading to increased plant biomass in G. max (Sirhindi et al. 2016), and as well as this result reported in G. max plants under Cd-stress (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, leading to improved growth traits in G. max (Mir et al. 2018). Likewise, Sirhindi et al. (2015) reported that the external use of JA in soybean plants exposed to Ni-toxicity cause restricted Ni uptake via roots and inhibited Ni interference with other necessary ionic metals needed for physio-biochemical processes, which 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-mediated regulation of main plant-metabolic processes reduced abiotic stress such as heavy metals (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 use of SA and JA in Ni-exposure plants probably restricts Ni translocation root-to-shoot by preventing Ni absorption and Ni storage in roots of plants [by chelating Ni to ligands including amino acids such as histidine (His), organic acids such as citrate, and nicotinamain (NA) and accumulating them into the vacuoles of root cells] and ameliorating Ni-toxicity effects resulting in biomass accretion (Zaid et al. 2019; Mubarak et al. 2021; Raza et al. 2021b).

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 and NM A. inflatum plants is considerably reduced in plant 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. The same results were obtained in proline contents of shoots of M and NM populations A. inflatum (Najafi-kakavand et al. 2019). Similarly, 100 μM Ni-induced proline accumulation was observed 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 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 physio-biochemical responses are probably in the response of M and NM A. inflatum populations to Ni-toxicity, and the signaling cascade mediated JA and SA.

Nickel stress considerably reduced proteins’ levels in several plant species, owing to decreased protein synthesis and hydrolyze (Hassan et al. 2019). Reduction of protein levels due to stress of heavy metals such as Ni in plants occurs 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 both populations of A. inflatum treated with high Ni concentration (400 μM) compared to the plants under moderate dose of Ni (200 μM) treatment. However, the supplement of SA, JA, and/or SA + JA with Ni in NM A. inflatum population showed promotion of the total protein levels in comparison to Ni-treated plants alone; nevertheless, these results were observed in the M population, except for JA treatments. Changes in the protein content of the shoots of M and NM populations of A. inflatum showed a similar trend with the root part (Najafi-kakavand et al. 2019). 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 plant growth regulator (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 and 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 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, H2O2 content enhanced, and also SOD, POD, CAT, and APX activities improved in both M and NM populations; however, APX activity did not change in the M population. According to our previous report, a similar trend in enzymatic activities (SOD, CAT, POD, and AXP) in response to Ni stress-induced oxidative stress was observed in the shoots of both A. inflatum populations. Unlike roots, the shoots AXP activity of the NM population did not indicate significant differences with the control plant (Najafi-kakavand et al. 2019). 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, showing an increasing trend in SOD, CAT, and POD activities in roots (Zhang et al. 2007). Also, O. sativa was exposed to different doses of Ni; increasing the concentration of Ni enhanced the H2O2 level in the roots, which subsequently reduced 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 H2O2 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 H2O2 levels than roots of plants without treatments. However, APX activity decreased in the M population, except at low concentrations of SA and JA, which showed an increase in APX activity at the highest dose of Ni. Likewise, our previous report on the activity of four enzymes in shoots of M and NM populations of A. inflatum under Ni stress showed that external application of SA (50 and 200 μM) and/or JA (5 and 10 μM) in these plants with increasing Ni concentration to 400 μM, the activities of SOD, POD, CAT, and AXP enzymes increased compared to control plants. However, application of SA and/or JA in the NM population exposed to different Ni concentrations, the shoot AXP activity did not display a significant change compared to the control (Najafi-kakavand et al. 2019).
decrease in APX activity may be due to the direct binding of Ni to the cysteine residue at the enzyme active site, leading the blocking of enzyme activity (Najafi-kakavand et al. 2019). 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 H₂O₂. Furthermore, in barley plants exposed to Cd-toxicity, the CAT and APX activities were enhanced, while the activities of these enzymes were 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 research has proved that SA can regulate genes expression level involved in encoding antioxidative defense mechanisms, thereby ameliorating 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 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 NiCl₂ (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 structure of the macromolecules such as proteins as well as cell membranes by reducing the content of H₂O₂ and malondialdehyde in plant cells. (Emamverdian et al. 2020). The current study determined that the external use of SA + JA resulted in a significantly reduced H₂O₂ content by enhancing antioxidative enzyme activity in the roots of both M and NM populations. However, the high activity of the APX enzyme in the M-population’s root was observed only along with the lower doses of SA and JA. Also, the activity of SOD, CAT, POD, and AXP enzymes was increased in shoots of two populations of A. inflatum exposed to simultaneous SA + JA treatments in plants under 400 μM Ni compared to plants treated with 400 μM Ni stress (Najafi-kakavand et al. 2019). 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, 2021b). These investigations illustrated that external SA and JA applications 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 effects 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 has 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 M and 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 interaction mechanisms between heavy metals and hormones affecting abiotic stresses such as SA and JA are not yet crystal clear and need further investigation.

Acknowledgements

We would like to thank the research deputy of Kermanshah University of Medical Sciences (Project Number 990252; Ethical Code IR.KUMS.REC.1399.321) and Razi University for providing the necessary facilities for carrying out this research.

Author 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.

Funding

Not applicable.

Data Availability

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

Declarations

Conflict of interest

The authors declare that they have no competing interests.

Ethics Approval

Not applicable.

Consent to Participate

Not applicable.

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