# Effects of Exogenous Salicylic Acid on Root Exudates of Jerusalem Artichoke under Aluminum Stress (Postprint)

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## Abstract

To investigate the effects of aluminum stress on Jerusalem artichoke root exudates and the alleviating role of exogenous salicylic acid, aluminum-tolerant Nanjing Jerusalem artichoke and aluminum-sensitive Ziyang Jerusalem artichoke were employed as experimental materials. Using a soil culture method at an aluminum concentration of 500 $\text{mol} \cdot \text{L}^{-1}$, we analyzed the effects of different SA concentrations (10, 100, and 1,000 $\text{mol} \cdot \text{L}^{-1}$) on organic acids, amino acids, and root tip-related metabolic enzyme activities in Jerusalem artichoke root exudates under aluminum stress. The results demonstrated that aluminum stress alone increased the concentrations of citric acid, oxalic acid, and malic acid in Jerusalem artichoke root exudates, with more pronounced increases in Nanjing Jerusalem artichoke than in Ziyang Jerusalem artichoke. Citrate synthase and malate dehydrogenase activities were enhanced under aluminum stress alone; proline content was significantly elevated, whereas total amino acid concentrations were significantly reduced. Following exogenous SA application, the concentrations of citric acid, oxalic acid, and malic acid secreted by Nanjing Jerusalem artichoke roots were all increased to varying degrees. However, oxalic acid secretion by Ziyang Jerusalem artichoke roots was significantly decreased after treatment with high-concentration (1,000 $\text{mol} \cdot \text{L}^{-1}$) SA, and malic acid concentrations remained essentially unchanged across all SA treatments. Citrate synthase activity was enhanced to varying degrees, while showing minimal effect on malate dehydrogenase activity in Nanjing Jerusalem artichoke root tips; high-concentration (1,000 $\text{mol} \cdot \text{L}^{-1}$) SA treatment significantly reduced malate dehydrogenase activity in Ziyang Jerusalem artichoke root tips. Proline content decreased significantly. In terms of total amino acid concentration changes, the maximum alleviating effect was observed in Nanjing Jerusalem artichoke under high-concentration (1,000 $\text{mol} \cdot \text{L}^{-1}$) SA and in Ziyang Jerusalem artichoke under low-concentration (10 $\text{mol} \cdot \text{L}^{-1}$) SA. Therefore, Jerusalem artichoke copes

with aluminum toxicity through organic acid secretion, and exogenous SA can promote the metabolic rate of organic acids in Jerusalem artichoke roots, leading to increased organic acid secretion to alleviate aluminum stress. This alleviating effect was more pronounced in the relatively more aluminum-tolerant Nanjing Jerusalem artichoke.

## Full Text

# Effects of Exogenous Salicylic Acid on Root Exudates of *Helianthus tuberosus* Under Aluminum Stress

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### Abstract

To investigate the effects of aluminum stress on root exudates of *Helianthus tuberosus* and the alleviating effect of exogenous salicylic acid (SA), we used aluminum-tolerant Nanjing *H. tuberosus* and aluminum-sensitive Ziyang *H. tuberosus* as experimental materials. Using a soil culture method with an aluminum concentration of 500 $\text{mol} \cdot \text{L}^{-1}$, we analyzed the effects of different SA concentrations (10, 100, and 1,000 $\text{mol} \cdot \text{L}^{-1}$) on organic acids, amino acids, and related metabolic enzyme activities in root tips of *H. tuberosus* under aluminum stress. The results showed that aluminum stress alone increased the concentrations of citric acid, oxalic acid, and malic acid in root exudates, with a greater increase observed in Nanjing *H. tuberosus* than in Ziyang *H. tuberosus*. Citrate synthase and malate dehydrogenase activities were enhanced under aluminum stress, while proline content increased significantly and total amino acid concentrations decreased markedly. Following exogenous SA application, the concentrations of citric acid, oxalic acid, and malic acid secreted by Nanjing *H. tuberosus* roots increased to varying degrees. However, in Ziyang *H. tuberosus*, oxalic acid secretion decreased significantly after treatment with high-concentration SA (1,000 $\text{mol} \cdot \text{L}^{-1}$), and malic acid concentrations showed no significant changes across all SA treatments. Citrate synthase activity was enhanced to different degrees, though SA had minimal effect on malate dehydrogenase activity in Nanjing *H. tuberosus* root tips while significantly reducing it in Ziyang *H. tuberosus* at high concentration. Proline content decreased significantly after SA treatment. In terms of total amino acid concentration, the maximum alleviating effect was achieved with high-concentration SA (1,000 $\text{mol} \cdot \text{L}^{-1}$) for Nanjing *H. tuberosus* and low-concentration SA (10 $\text{mol} \cdot \text{L}^{-1}$) for Ziyang *H. tuberosus*. These findings indicate that *H. tuberosus* responds to aluminum toxicity by secreting organic acids, and that exogenous SA can promote organic acid metabolism in the root system to secrete more organic

acids for aluminum stress alleviation, with this effect being more pronounced in the relatively aluminum-tolerant Nanjing cultivar.

**Keywords:** *Helianthus tuberosus*, red soil region, aluminum stress, salicylic acid, root exudates

## Introduction

Red soil, the zonal soil of southern China, accounts for nearly one-quarter of the country' s total land area. Characterized by low pH values (4.0–5.5), low organic matter content, and limited exchangeable base cations, red soils have experienced intensifying acidification due to frequent acid rain in southern China, which mobilizes substantial amounts of previously insoluble aluminum. The resulting increase in soluble aluminum content significantly enhances soil aluminization, severely affecting root development and the absorption of water and nutrients, and is widely recognized as a primary factor limiting crop growth in acidic soils.

Plants employ two main aluminum tolerance mechanisms: external exclusion and internal tolerance. Through external exclusion, plant roots secrete organic acids, phenolic compounds, and phosphates that modify the rhizosphere environment by chelating aluminum ions into non-toxic or less toxic compounds, thereby enhancing internal aluminum absorption, transport, and tolerance while reducing aluminum toxicity. Additionally, plants regulate tolerance through transcriptional control of specific genes. Studies have identified ART1 and STOP1 as important regulators of aluminum tolerance, while transcription factors WRKY46 and ASR5 operate through different mechanisms—WRKY46 participates in osmotic stress responses and stomatal regulation, whereas ASR5 binds to the promoter region of the STAR1 gene to enhance its expression and improve rice aluminum tolerance.

*Helianthus tuberosus* (Jerusalem artichoke), a perennial herbaceous plant in the family Compositae and genus *Helianthus*, exhibits strong adaptability to various ecological environments and is widely cultivated globally. Rich in inulin, Jerusalem artichoke serves as an excellent source for ethanol production, biological fermentation, and oil extraction, while also possessing medicinal properties such as heat-clearing and diuretic effects. To expand its cultivation in southern China, addressing the impact of aluminum stress on Jerusalem artichoke growth has become an urgent priority. Investigating organic acids in root exudates and related metabolic enzyme activities will help elucidate the plant' s response to aluminum stress.

Salicylic acid (SA), a phenolic compound ubiquitous in plants, functions as a signaling molecule in stress responses by inducing pathogenesis-related protein gene expression to confer systemic acquired resistance. Numerous studies have demonstrated its important role in alleviating both biotic and abiotic stresses.

For instance, Ma et al. (2020) found that exogenous SA enhanced antioxidant capacity in wild jujube seedlings under salt stress, mitigating salt damage and improving net photosynthetic rate and growth. Cao et al. (2015) reported that exogenous SA improved antioxidant enzyme activity and photosynthetic efficiency in *H. tuberosus* under aluminum stress. However, how organic acids and amino acids in Jerusalem artichoke root tips respond to aluminum stress conditions, and what role exogenous SA plays in this process, remain important questions. This study analyzes citric acid, malic acid, oxalic acid, proline, amino acids, and the activities of citrate synthase and malate dehydrogenase in *H. tuberosus* root exudates under aluminum stress, aiming to provide a scientific basis for the safe cultivation and application of Jerusalem artichoke in red soil regions with high aluminum content.

## Materials and Methods

### 1.1 Experimental Materials

Two Jerusalem artichoke cultivars were selected: aluminum-tolerant Nanjing *H. tuberosus* and aluminum-sensitive Ziyang *H. tuberosus*.

### 1.2 Experimental Methods

Uniformly sized Jerusalem artichoke tubers were cultivated in soil and placed in a light incubator for germination (day: 30°C, 90% relative humidity, 60% light intensity, 14 h; night: 26°C, 80% relative humidity, 0% light, 10 h). When shoots reached approximately 8 cm in height, uniformly growing seedlings were transplanted into perforated plastic pots (3 plants per pot, 3 replicates per group).

$Al^{3+}$ was applied as $AlCl_3 \cdot 6H_2O$ mixed with complete nutrient solution and evenly sprayed onto the root zone soil at a concentration of 500 $mol \cdot L^{-1}$. The treatment solution pH was adjusted to 4.5 using diluted HCl. SA solution was sprayed evenly on both leaf surfaces daily for 7 days, after which root exudates were collected. Concentrations were determined based on preliminary experiments, with experimental groups detailed in Table 1 .

**Root exudate collection:** Healthy, intact roots were rinsed 3–4 times with distilled water, blotted dry with filter paper, and immersed in 200 mL of 0.5 $mmol \cdot L^{-1}$ $CaCl_2$ solution (3 seedlings per treatment). The beaker bottom was wrapped with aluminum foil to exclude light, and exudates were collected under illumination for 6 h. Roots were then rinsed with 100 mL deionized water, yielding 300 mL total exudate solution. This was concentrated to 25 mL using a rotary evaporator at 40°C, filtered through a 0.45 μm aqueous membrane, and stored at -20°C for subsequent analyses.

**Crude enzyme extraction:** Two-centimeter root tips (0.1 g) were ground in a mortar on ice with 1 mL extraction buffer containing 100 $mmol \cdot L^{-1}$ Tris-HCl buffer (pH 8.0), 0.1% (V/V) Triton X-100, 2% (W/V) PVP, and 10 $mmol \cdot L^{-1}$

isoascorbic acid. The homogenate was centrifuged at 15,000 $r \cdot min^{-1}$ for 5 min at 4°C, and the supernatant was used for enzyme activity assays.

### 1.3 Index Determination

Proline content was determined using the acidic ninhydrin colorimetric method. Citric acid, oxalic acid, malic acid, and amino acid concentrations were measured by high-performance liquid chromatography (HPLC). Citrate synthase activity was assayed by mixing 20 µL crude enzyme extract with 1 mL reaction solution [100 $mmol \cdot L^{-1}$ Tris-HCl buffer (pH 8.0), 5 $mmol \cdot L^{-1}$ $MgCl_2$, 0.5 $mmol \cdot L^{-1}$ DTNB, 0.15 $mmol \cdot L^{-1}$ acetyl-CoA, and 4 $mmol \cdot L^{-1}$ oxaloacetic acid; DTNB was light-protected]. Activity was determined by measuring absorbance changes at 412 nm every 30 s for 3 min. Malate dehydrogenase activity was measured by adding 20 µL crude enzyme extract to 1 mL reaction solution (100 $mmol \cdot L^{-1}$ Tris-HCl buffer pH 8.0, 0.5 $mmol \cdot L^{-1}$ EDTA-$Na_2$, 0.2 $mmol \cdot L^{-1}$ NADH, 70 $mmol \cdot L^{-1}$ KCl) and initiating the reaction with 1 mL of 1 $mmol \cdot L^{-1}$ oxaloacetic acid. Absorbance at 340 nm was recorded every 30 s for 3 min, with NADH consumption or production used as the evaluation standard.

### 1.4 Data Processing

All measurements were performed in triplicate, and means ± standard errors were calculated. Data were analyzed using Duncan' s multiple range test in SPSS 22.0 software, and figures were prepared using Origin 8.5.

## Results

### 2.1 Effects of Exogenous SA on Citric Acid Concentration in Root Exudates Under Aluminum Stress

As shown in Figure 1 [Figure 1: see original paper], aluminum treatment alone (T1) significantly increased citric acid concentration in Nanjing *H. tuberosus* root exudates by 2.78-fold ($P<0.05$), whereas the increase in Ziyang *H. tuberosus* was only 14.78% and not statistically significant ($P>0.05$), indicating differential responses between the two cultivars. Citric acid concentrations in both cultivars increased progressively with SA concentration. For Nanjing *H. tuberosus*, T4 (1,000 $mol \cdot L^{-1}$ SA) showed a 3.32-fold increase compared to T1 ($P<0.05$), while Ziyang *H. tuberosus* exhibited a 6.63-fold increase under the same conditions ($P<0.05$). These results demonstrate that SA can modulate citric acid concentrations in Jerusalem artichoke root exudates to cope with aluminum stress, with more pronounced alleviation effects in the aluminum-sensitive Ziyang cultivar.

### 2.2 Effects of Exogenous SA on Oxalic Acid Concentration in Root Exudates Under Aluminum Stress

Figure 2 [Figure 2: see original paper] reveals that aluminum treatment alone (T1) significantly increased oxalic acid concentration in root exudates ($P<0.05$). Following exogenous SA application, Nanjing *H. tuberosus* showed increasing oxalic acid concentrations with rising SA levels, peaking at 1,000 $\text{mol} \cdot \text{L}^{-1}$ SA (T4) with a 192.69% increase over T1 ($P<0.05$). In contrast, Ziyang *H. tuberosus* reached maximum oxalic acid content at 100 $\text{mol} \cdot \text{L}^{-1}$ SA (T3), showing a 2.44-fold increase ($P<0.05$), but exhibited significantly lower oxalic acid content at 1,000 $\text{mol} \cdot \text{L}^{-1}$ SA compared to T1 ($P<0.05$). This suggests that excessively high SA concentrations may inhibit oxalic acid secretion in sensitive cultivars.

### 2.3 Effects of Exogenous SA on Malic Acid Concentration in Root Exudates Under Aluminum Stress

As illustrated in Figure 3 [Figure 3: see original paper], aluminum treatment alone (T1) did not significantly alter malic acid concentrations in either cultivar compared to the control. After SA application, Nanjing *H. tuberosus* reached maximum malic acid concentration at 10 $\text{mol} \cdot \text{L}^{-1}$ SA, representing a 2.15-fold increase over T1 ($P<0.05$). Ziyang *H. tuberosus* also peaked at this concentration, though the increase was not statistically significant ($P>0.05$).

### 2.4 Effects of Exogenous SA on Citrate Synthase and Malate Dehydrogenase Activities in Root Tips Under Aluminum Stress

Table 2 shows that in the control group (T0), both citrate synthase and malate dehydrogenase activities were significantly lower in Nanjing *H. tuberosus* than in Ziyang *H. tuberosus* ($P<0.05$). Aluminum treatment alone (T1) enhanced citrate synthase activity in both cultivars. Exogenous SA at 10, 100, and 1,000 $\text{mol} \cdot \text{L}^{-1}$ further increased citrate synthase activity to varying degrees, though in Ziyang *H. tuberosus* this effect was only significant at high SA concentration (T4). Nanjing *H. tuberosus* achieved maximum citrate synthase activity at 10 $\text{mol} \cdot \text{L}^{-1}$ SA, showing a 1.46-fold increase over T1 ($P<0.05$). Conversely, different SA concentrations had minimal effect on malate dehydrogenase activity in Nanjing *H. tuberosus* but significantly reduced it in Ziyang *H. tuberosus*, with the lowest activity (T4) decreasing by 53.19% compared to the highest (T1) ($P<0.05$).

### 2.5 Effects of Exogenous SA on Proline Content in Root Exudates Under Aluminum Stress

Figure 4 [Figure 4: see original paper] demonstrates that aluminum stress alone (T1) significantly increased proline content in both cultivars, with increases of 3.79% and 3.08% respectively ($P<0.05$). Following exogenous SA application, proline content decreased significantly compared to T1 ($P<0.05$). The maximum reduction in Nanjing *H. tuberosus* occurred at 100 $\text{mol} \cdot \text{L}^{-1}$ SA (T3), with a

5.11% decrease ($P<0.05$), while Ziyang *H. tuberosus* showed the greatest decline at 1,000 $mol \cdot L^{-1}$ SA (T4), decreasing by 4.48% ($P<0.05$).

### 2.6 Effects of Exogenous SA on Amino Acid Concentration in Root Exudates Under Aluminum Stress

Under aluminum treatment alone (T1), total amino acid concentrations in root exudates decreased substantially in both cultivars, indicating that 500 $mol \cdot L^{-1}$ aluminum ion concentration impairs amino acid secretion. Nanjing *H. tuberosus* showed a particularly significant reduction of 89.59% ($P<0.05$). Exogenous SA application increased total amino acid concentrations in Nanjing *H. tuberosus*, with high-concentration SA (1,000 $mol \cdot L^{-1}$) producing a significant elevation ($P<0.05$). In contrast, Ziyang *H. tuberosus* showed the opposite trend, with low-concentration SA (10 $mol \cdot L^{-1}$) producing the most significant increase and maximum alleviation effect compared to T1 ($P<0.05$).

## Discussion and Conclusion

Previous research has demonstrated that organic acid secretion under aluminum stress correlates positively with aluminum concentration. Studies on aluminum-induced citric acid secretion in soybean (*Glycine max*) and *Trichosanthes kirilowii* have shown increased citric acid secretion under aluminum stress, a pattern confirmed in our experiments. We further observed that appropriate SA supplementation induces favorable changes in organic acids and related metabolic enzymes. Compared to aluminum treatment alone (T1), citric acid content increased significantly with SA concentration, indicating that exogenous SA regulates citric acid synthesis and metabolism in Jerusalem artichoke roots.

Notably, oxalic acid exhibited different responses between cultivars: the aluminum-tolerant Nanjing *H. tuberosus* showed continuously increasing oxalic acid secretion with rising SA concentrations, while the aluminum-sensitive Ziyang *H. tuberosus* displayed a "low promotion, high inhibition" pattern. This suggests that excessively high SA concentrations may not provide positive regulation for aluminum tolerance in sensitive cultivars, consistent with Liu (2011) who found that low-concentration SA (10 $mol \cdot L^{-1}$) significantly alleviated aluminum inhibition of root elongation and reduced aluminum accumulation in root tips, whereas high SA concentrations caused dual stress rather than alleviating aluminum toxicity.

Our results indicate that rapid increases in the three organic acids under aluminum stress help reduce aluminum damage to root tips, and that exogenous SA modulates the response of organic acid metabolism and related enzymes to aluminum stress, ultimately leading to organic acid accumulation for stress alleviation. This aligns with Kong (2013) regarding hydrogen peroxide and SA signaling interactions in soybean aluminum tolerance. Exogenous SA at appropriate concentrations alleviates aluminum damage, likely by affecting cor-

responding enzymes and altering organic acid secretion metabolism, thereby enhancing aluminum tolerance.

Previous studies have established that aluminum-tolerant wheat secretes 5–10 times more malic acid from root tips than sensitive varieties, and that organic acid secretion correlates positively with aluminum tolerance across 36 wheat cultivars. Our comparison of the two Jerusalem artichoke cultivars revealed greater organic acid secretion from Nanjing *H. tuberosus* under aluminum stress, confirming that aluminum-tolerant plants secrete more organic acids to cope with adverse conditions.

Citrate synthase and malate dehydrogenase are key enzymes in biological metabolism. Citrate synthase catalyzes the first reaction of the tricarboxylic acid (TCA) cycle, converting acetyl-CoA and oxaloacetic acid to citrate, while malate dehydrogenase catalyzes the reversible reaction between malic acid and oxaloacetic acid, representing another important TCA cycle enzyme. Plants enhance citrate synthase expression to cope with stress conditions. Our results show enhanced citrate synthase activity under aluminum stress, with different SA concentration requirements between cultivars—Nanjing *H. tuberosus* required much lower SA concentrations than Ziyang *H. tuberosus*—while citric acid content generally increased under catalysis by this enzyme.

Increased malate dehydrogenase gene expression can enhance plant aluminum tolerance, indicating that malate dehydrogenase activity directly affects aluminum resistance. Our findings show that aluminum stress and exogenous SA treatment significantly affected Ziyang *H. tuberosus* but not the more tolerant Nanjing cultivar, suggesting that the latter' s enhanced malate dehydrogenase activity under aluminum toxicity promotes rapid organic acid secretion for aluminum chelation, thereby improving aluminum tolerance.

Amino acids enhance plant adaptation to abiotic stress by participating in physiological metabolism modifications or regulating gene expression and key enzyme activities. Under aluminum stress alone, proline content increased significantly in both cultivars, consistent with studies on cold resistance in *Zoysia* grass, indicating that plants maintain cellular osmotic balance under stress by increasing proline content. Exogenous SA application reduced proline content, confirming stress alleviation. Total amino acid concentrations decreased significantly under aluminum stress, possibly due to root damage from high aluminum concentrations or differential effects on individual amino acid components. Although SA application increased amino acid concentrations, levels remained below normal, suggesting that Jerusalem artichoke primarily relies on organic acid secretion rather than amino acids for aluminum detoxification at 500 $\mu mol \cdot L^{-1}$ aluminum concentration. This finding is supported by research on evergreen poplar root exudates under aluminum stress.

In conclusion, aluminum stress significantly affected organic acids, amino acids, and related indicators in Jerusalem artichoke root exudates. Aluminum induced organic acid secretion, which is closely associated with stress responses. Exoge-

nous SA application increased citric acid, oxalic acid, and malic acid concentrations compared to aluminum treatment alone, with more pronounced effects in Nanjing *H. tuberosus*, except for the "low promotion, high inhibition" effect on oxalic acid in Ziyang *H. tuberosus*. Total amino acid concentrations decreased under aluminum stress but increased after SA application. Overall, appropriate concentrations of exogenous SA alleviate aluminum stress by promoting greater organic acid secretion from roots, with more effective alleviation observed in the aluminum-tolerant Nanjing cultivar.

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*Note: Figure translations are in progress. See original paper for figures.*