Ciencia Nicolaita 96: 66-75; Abril de 2026 https://doi.org/10.35830/cn.vi96.907

Azotobacter vinelandii AEIV volatiles protect *Arabidopsis* seedlings from zinc damage of roots via an abscisic acid crosstalk

Paola Peralta López, Karina Alejandra Balderas Ruíz, Gipumi Torres Abe, Carla Sánchez Arana, Cinthia Nuñez, Ángel Arturo Guevara García

Instituto de Biotecnología, Uiversidad Nacional Autonoma de México. Av. Universidad 2001, Chamilpa, 62210. Cuernavaca, Morelos.

Abstract

Heavy metal pollution (i.e., lead, arsenic, copper, zinc) causes stress in plants, affecting their growth and development. *Azotobacter vinelandii* strains have attracted considerable interest due to their ability to fix nitrogen, produce biodegradable biopolymers, and promote plant growth. In this study, the effect of high zinc (Zn) concentrations on *Arabidopsis thaliana* growth *in vitro*, alone and in interaction with *A. vinelandii* AEIV was evaluated. Notably, the communication mediated by bacterial-emitted volatiles exerted a protective effect against Zn-induced damage in internal root tissues and influenced the abscisic acid (ABA) response. These findings suggest that *A. vinelandii* AEIV helps the plant to cope with the damaging effects of heavy metals and influences ABA signaling pathways.

Keywords: *Arabidopsis thaliana*, *Azotobacter vinelandii* AEIV, plant-microbe interaction, heavy metals, abscisic acid.

Introduction

Plants, as sessile organisms, are unable to escape unfavorable environmental conditions, therefore, they remain at the site of germination until life cycle completion under adverse conditions such as drought, pollution, or diseases caused by phytopathogens. To survive and adapt, plants have evolved complex tolerance mechanisms that are further supported by the interactions with microorganisms colonizing leaves, flowers, and especially roots, or inhabiting internal tissues as endophytes. Although some of these microorganisms may be pathogenic, most are beneficial, contributing to growth under limiting conditions and/or strengthening the immune system (Pantigoso *et al.,* 2022).

Plant Growth-Promoting Bacteria (PGPB) act as probiotic microorganisms via multiple mechanisms, including enhanced nutrient uptake, atmospheric nitrogen (N) fixation, and the production of antimicrobial compounds (Hasan *et al.,* 2024). PGPB therefore represent a promising alternative for achieving sustainable agriculture under increasingly restrictive environmental conditions such as nutrient deficiency. Nitrogen (N) is a macronutrient required in large amounts by plants owing to its essential role as a constituent of amino acids, nucleic acids and a wide range of metabolites. However, its availability in soils is often limited, leading to the widespread use of fertilizers to sustain agricultural productivity (Barney, 2024).

N-fixing bacteria such as *Azotobacter vinelandii* may contribute to the nutritional requirements of crops (Yoshida *et al.,* 2022). In addition, this bacterium produces plant growth-regulating compounds (phytohormones), including auxins, gibberellins, and cytokinins (Aasfar *et al.,* 2021), and mitigates damage caused by heavy-metal toxicity and high soil salinity (Das, 2019; Sahoo *et al.,* 2021).

Plant responses to biotic and abiotic stressors are complex and multifactorial. In this context, *Arabidopsis thaliana* has been helpful towards elucidating diverse biological processes and generating knowledge that can be extrapolated to crop species. This species, which is closely related to mustard and cauliflower, has several advantageous characteristics, including a small size, a short life cycle, and high seed production. In addition, *A. thaliana* possesses a small, fully annotated genome, which

Dates
Received: February 10, 2026
Accepted: March 28, 2026
Available online: April 2026

Corresponding author
Ángel Arturo Guevara García
email: arturo.guevara@ibt.unam.mx
ORCID: 0000-0002-5910-0255

Cite this article
Peralta-López P, Balderas-Ruíz KA, Torres-Abe G, Sánchez-Arana C, Nuñez C, Guevara-García AA (2026). *Azotobacter vinelandii* AEIV volatiles protect *Arabidopsis* seedlings from zinc damage of roots via an abscisic acid crosstalk. *Ciencia Nicolaita* 96:66-75. DOI:10.35830/cn.vi96.907

facilitates genetic analyses (https://abrc.osu.edu/) (Ferjani *et al.,* 2023). Among the available genetic resources, reporter lines are particularly valuable for studying genes, proteins, and regulatory processes involved in stress responses (Miki y McHugh, 2004).

Heavy metals may be present naturally in most ecosystems; however, their accumulation as a result of anthropogenic activities presents serious environmental and health risks because they are neither chemically nor biologically degradable and tend to move through trophic chains (Shaffique *et al.,* 2023). Metals such as arsenic (As), lead (Pb), and mercury (Hg) have no known biological function and are toxic even at low concentrations. In contrast, others, such as copper (Cu) and zinc (Zn), are essential micronutrients at nanomolar or micromolar concentrations but become toxic to plants when present in excess (Nakayama *et al.,* 2020). Optimal Zn concentrations in plant tissues typically range from 15 to 50 mg/kg dry weight, however, mining and metallurgical activities can increase Zn levels to more than 400 mg/kg dry weight in contaminated soils (Bazihizina *et al.,* 2014). Such elevated concentrations inhibit plant growth, induce leaf chlorosis, and promote oxidative damage (Stuiver *et al.,* 2014; Gong *et al.,* 2020).

Proper plant development and adaptation to stress depend on the coordinated balance among multiple phytohormones and signaling pathways. Auxins, cytokinins, and gibberellins orchestrate growth and development, whereas jasmonic acid and salicylic acid mediate responses to biotic stress. In contrast, abscisic acid (ABA) is primarily associated with tolerance to abiotic stress. ABA is synthesized in chloroplasts, where photosynthesis occurs, and this process is directly affected by heavy-metal stress. This relationship suggests that reduced photosynthetic activity may trigger stress-responsive gene expression (Ng *et al.,* 2014; Felemban *et al.,* 2019; Lim *et al.,* 2022). Based on this framework, we hypothesized that *A. vinelandii* AEIV is a PGPB capable of enhancing plant tolerance to high Zn levels. To test this hypothesis, our objectives were: (1) to establish a reliable plant–bacterium–heavy metal interaction system and (2) to explore the role of phytohormones in the response to Zn-induced abiotic stress.

Materials and methods

Biological material and chemical reagents

Wild-type *A. thaliana* seeds from Columbia (Col-0) and Wassilewskija (Ws) ecotypes were used, together with the ABA-insensitive mutant line *abi5*, which lacks expression of the ABI5 transcription factor (Finkelstein *et al.,* 2000), as well as the ABA reporter lines *pABI5::GUS* (López-Molina *et al.,* 2001) and the cellular damage reporter line *pERF115::GUS:GFP* (Heyman *et al.,* 2016). The bacterial strain used in this study was *A. vinelandii* AEIV (Larsen y Haugh 1971; Nuñez *et al.*, 2013), which is deposited in a microbial collection (Colección Nacional de Cepas Microbianas y Cultivos Celulares, CINVESTAV) with the code CDBB-B-1529.

Chemical reagents included $ZnSO_4 \cdot 7H_2O$ (Química Meyer); yeast extract, peptone, and bactoagar (BD Bacto); sucrose, glucose, HCl, and ethanol (JT Baker); 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) and phytoagar (Phytotechnology); and ABA, NaH_2PO_4, Na_2HPO_4, EDTA, Triton™ X-100, $K_3Fe(CN)_6$, $K_4Fe(CN)_6$, KOH, and NaOH (Sigma-Aldrich).

Seed sowing and growth conditions

Seeds were disinfected with 96% (v/v) ethanol and 10% (v/v) sodium hypochlorite (commercial bleach) for 4 minutes each, with agitation at 25 °C. To remove residual chlorine, four consecutive washes with sterile deionized water were performed. To synchronize germination, seeds were stratified at 4 °C for 48 h in darkness. Seeds were sown on plates containing MS 0.2x medium solidified with phytoagar 1% (w/v), adjusted to pH 7.0 and supplemented with 0.6% (w/v) sucrose. For germination (3 days) and seedling growth (4 days), plates were sealed with *Parafilm* and incubated vertically in a growth chamber (Percival CU41L4) under conditions of 22 °C and a 16/8 h light/dark photoperiod.

The *A. vinelandii* AEIV strain was preserved at −80 °C in liquid PY medium (0.3% yeast extract, 0.5% peptone, and 2% sucrose (w/v)) supplemented with 25% (v/v) glycerol. For activation, the strain was inoculated into 3 mL of PY medium and incubated at 30 °C and 250 rpm for 24 h. Then, 100 µL of this culture were spread on a plate with PY medium solidified with bactoagar 1% (w/v) to isolate a single colony, which was used to prepare a liquid culture until the desired concentration was reached. Initially, bacterial inoculum concentrations of 1×10^5 to 1×10^9 cells/mL were tested, but since an inoculum of 1×10^5 showed limited growth, while all other concentrations (1×10^6, 1×10^7, 1×10^8 and 1×10^9) showed similar growth in the three plant-bacteria interaction systems, all reported experiments were performed with an inoculum of 1×10^8 cells/mL.

A. thaliana – A. vinelandii AEIV interaction systems

Three plant–bacterium interaction systems were implemented using Petri plates: (1) Direct Contact (DC), (2) Diffusible plus Volatile Compounds (DiVo), and (3) Volatile Compounds (CoVo). Briefly, Petri dishes were divided into three (DC) or two sections (DiVo) with a previously disinfected 2 cm wide glass plate, whereas factory-divided Petri dishes were used for CoVo. In all cases, the bacterium was grown on solid PY medium, while seedlings were grown on solid MS 0.2x medium. In all systems, the bacterial inoculum consisted of 10 µL of a culture containing 1×10^8 cells/mL of *A. vinelandii* AEIV (treatment) or 10 µL of liquid PY medium (control). Inoculated plates were incubated for 48 h at 30 °C to promote bacterial growth, after which 4-day-old *A. thaliana* seedlings were transferred to the interaction systems. Finally, plates were sealed with *Parafilm* and maintained vertically in a plant growth chamber (22 °C, 16 h light/8 h darkness photoperiod) for 6 additional days.

Zn and ABA assays

For Zn experiments, a 100 mM $ZnSO_4 \cdot 7H_2O$ stock solution was used to prepare 0.2x MS medium supplemented with 100, 200, and 300 µM of the heavy metal. For control treatments, the media were prepared with the corresponding microliters of the solvent solution equivalent to the highest Zn concentration. At the time of seedling transfer, the position of each root tip was marked, and the plates were incubated under the described growth conditions for 6 days, after which phenotypic parameters were measured. For the ABA experiment, 1 mL of a 100 mM stock solution was prepared using the same solvent employed for zinc sulfate, from which dilutions were made to obtain a final concentration of 15 µM.

Evaluation of phenotypic parameters

Nine phenotypic parameters were evaluated using 18 individuals at 10 days after germination (4 days at the time of transfer + 6 days of exposure to Zn with or without bacterial interaction), as follows: primary root growth (cm) and rosette diameter (cm) were measured using a ruler. The number of lateral roots and leaves was counted under a stereomicroscope (Olympus SZ40), and the length of the longest lateral root was measured. Biomass (fresh weight) was quantified using an analytical balance (Ohaus PX124), six seedlings per plate/treatment were used to record total biomass; roots were excised to obtain shoot biomass, and root biomass as total biomass − shoot biomass difference.

β-glucuronidase (GUS) histochemical activity

Seedlings were incubated overnight at 37 °C in reaction buffer [1 mg/mL X-Gluc; 50 mM NaH_2PO_4; 50 mM Na_2HPO_4; 10 mM EDTA; 0.1% (v/v) Triton™ X-100; 2 mM $K_3Fe(CN)_6$; 2 mM $K_4Fe(CN)_6$], with the pH adjusted to 7. To assess enzymatic activity, seedlings were transferred to 1 mL of acidic solution (0.2 M HCl; 20% (v/v) CH_3OH) and incubated for 50 minutes at 62 °C. The solution was replaced with 1 mL of basic solution (7% (w/v) NaOH; 60% (v/v) CH_3CH_2OH), where seedlings were kept for 20 minutes at room temperature. Subsequently, seedlings were subjected to successive washes with 40, 20, and 10% (v/v) ethanol solutions for 20 minutes each. As a final step, the 10% ethanol solution was replaced with 50% (v/v) glycerol for seedling preservation. Seedlings were mounted in manually designed chambers (glass slides, coverslips, *Parafilm*, and clear nail polish) and observed under a bright-field microscope (AmScope T490B) equipped with a camera to obtain digital images.

Results

In vitro plant-bacteria interaction systems

The effects of plant growth-promoting bacteria are highly dependent on the inoculation system. The interaction of *Arabidopsis* with *A. vinelandii* has not previously been analyzed; therefore, a combined culture medium suitable for both bacterial growth (PY) and plant growth (MS) was designed to evaluate bacterial and plant growth using three different interaction systems: Direct Contact (DC), Diffusible plus Volatile Compounds (DiVo), and Volatile Compounds (CoVo).

In the three interaction systems, the effects of the bacterium were evaluated on root biomass, shoot biomass, total biomass, leaf number, shoot diameter, primary root growth, number of lateral roots, and the length of the longest lateral root. In the DC system (**Figure 1a**), the bacterium did not exert a plant growth–promoting effect; instead, key growth parameters such as total biomass and primary root growth were significantly reduced in the presence of the bacterium (data not shown). This unexpected growth inhibition was most likely associated with excessive bacterial proliferation even overgrowing the root system (inoculated at 1×10^8 CFU/mL). Similarly, in the DiVo system (**Figure 1b**), *A. vinelandii* AEIV also failed to show a clear plant growth–promoting effect, as bacterial effects were negative for nearly all evaluated parameters, except for the number of lateral roots, which was the only parameter showing a statistically significant increase (data not shown).

In both the DiVo and CoVo systems, the unexpected inhibition (DiVo) and non-promotion (CoVo) of plant growth could be associated with the production of diffusible bacterial compounds that negatively affect root growth. The identification of the chemical compounds remains pending.

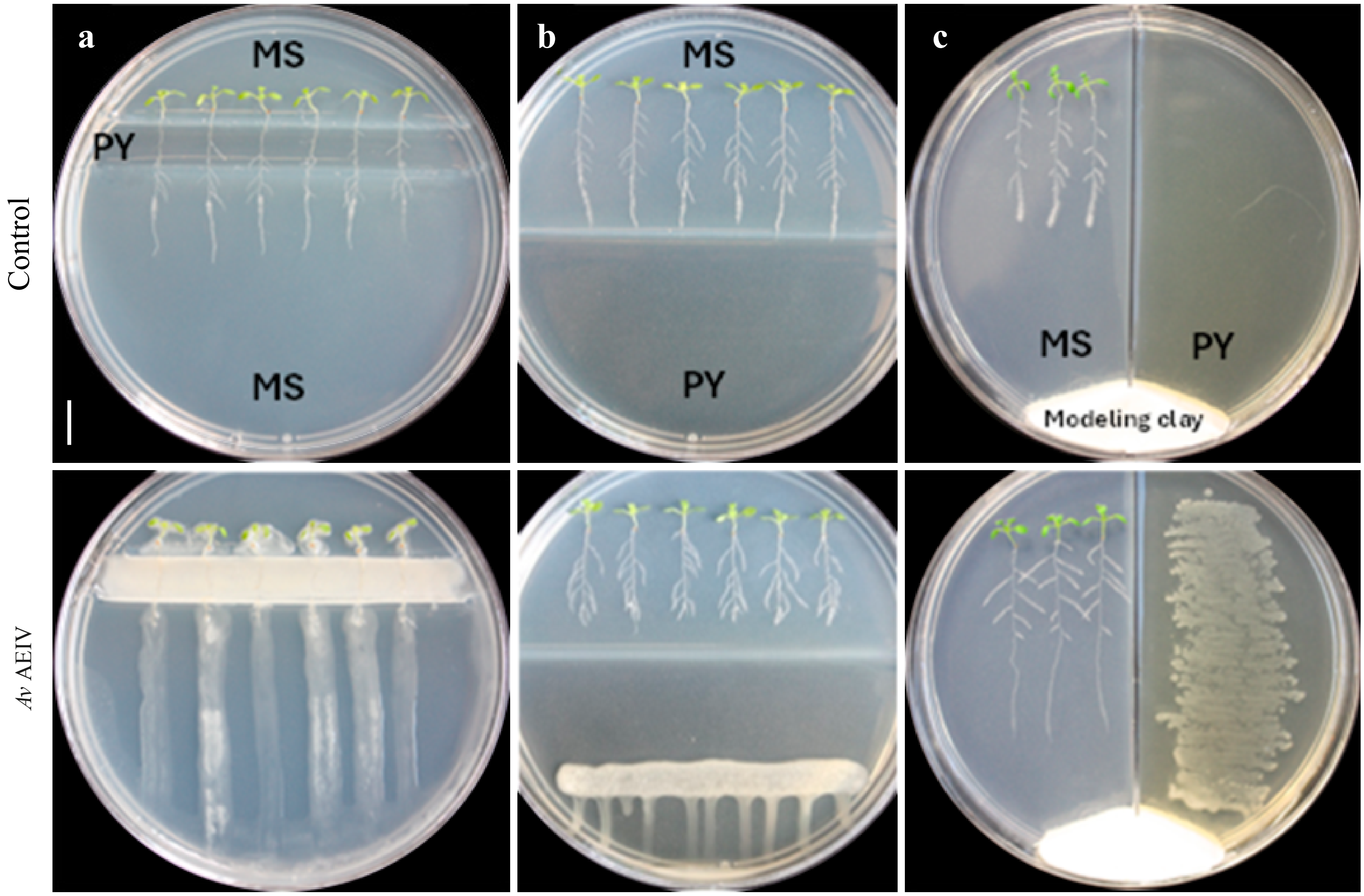

Figure 1. ***In vitro* interaction systems between *A. thaliana* and *A. vinelandii* AEIV.** (**a**) Direct Contact (DC), (**b**) Diffusible plus Volatile compounds (DiVo), and (**c**) Volatile Compounds (CoVo). Upper panels: control conditions; lower panels: interaction with *A. vinelandii* AEIV. MS: medium for *A. thaliana*; PY: medium for *A. vinelandii* AEIV. N = 18. Scale bar = 1 cm.

A. vinelandii AEIV produces VOCs that promote plant growth

In contrast, when bacterial growth on the plant medium was prevented, in the CoVo interaction system, the effects of *A. vinelandii* AEIV were neutral for some growth parameters, such as leaf number and shoot diameter, and even negative for others, such as the number of lateral roots (data not shown); however, some important parameters, including primary root growth and total biomass were positively and significantly affected (**Figure 2**).

These results suggest that interaction mediated by volatile compounds promoted plant growth, as the physical separation between both organisms allowed adequate bacterial growth in an optimal culture medium, while simultaneously preventing diffusion of growth-inhibitory compounds into the plant medium and eventual root colonization. Nevertheless, the differential effects observed among the interaction systems, together with the variable impact on individual growth parameters, highlight the complexity of plant–microorganism interactions, which are still far from being fully understood.

Effects of zinc on plant development

Evaluation of *A. thaliana* seedlings grown for 6 days on media supplemented with Zn confirmed that this heavy metal exerts a dose-dependent negative effect on biomass production and primary root growth (**Figure 3**), with statistically significant differences observed at $ZnSO_4$ concentrations between 100 and 300 µM. In addition, other growth parameters, including shoot diameter and the number of lateral roots, among others, were negatively affected (data not shown).

A. vinelandii AEIV produces volatiles that mitigate the cellular damage caused by Zn

To further explore the potential applications of *A. vinelandii* AEIV in promoting plant growth under adverse conditions, we investigated whether the volatile compounds produced by this strain influence the growth of *A. thaliana* under Zn stress. Seedling growth was evaluated in the CoVo interaction system under control conditions (without Zn and without bacteria) and under Zn exposure ($ZnSO_4$ 0 to 300 µM) in the presence of

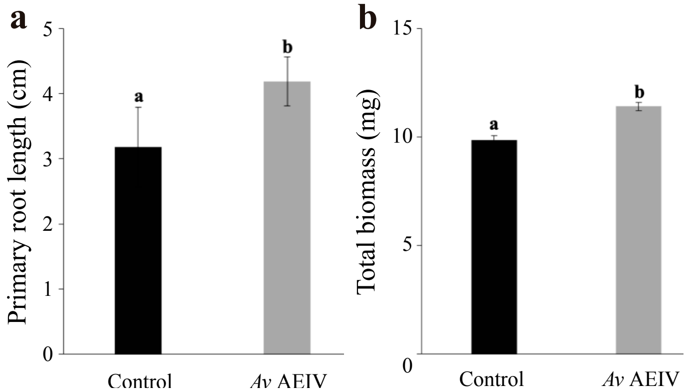

Figure 2. Effects of *A. vinelandii* AEIV volatiles on *A. thaliana* growth in the CoVo interaction system. (**a**) Primary root length (cm). (**b**) Total biomass (mg). Seedlings were grown under conditions in which bacterial growth on the plant medium was prevented. Data represent mean ± SE (N = 18). Different letters indicate statistically significant differences between treatments (Student's *t* test; $p \leq 0.05$).

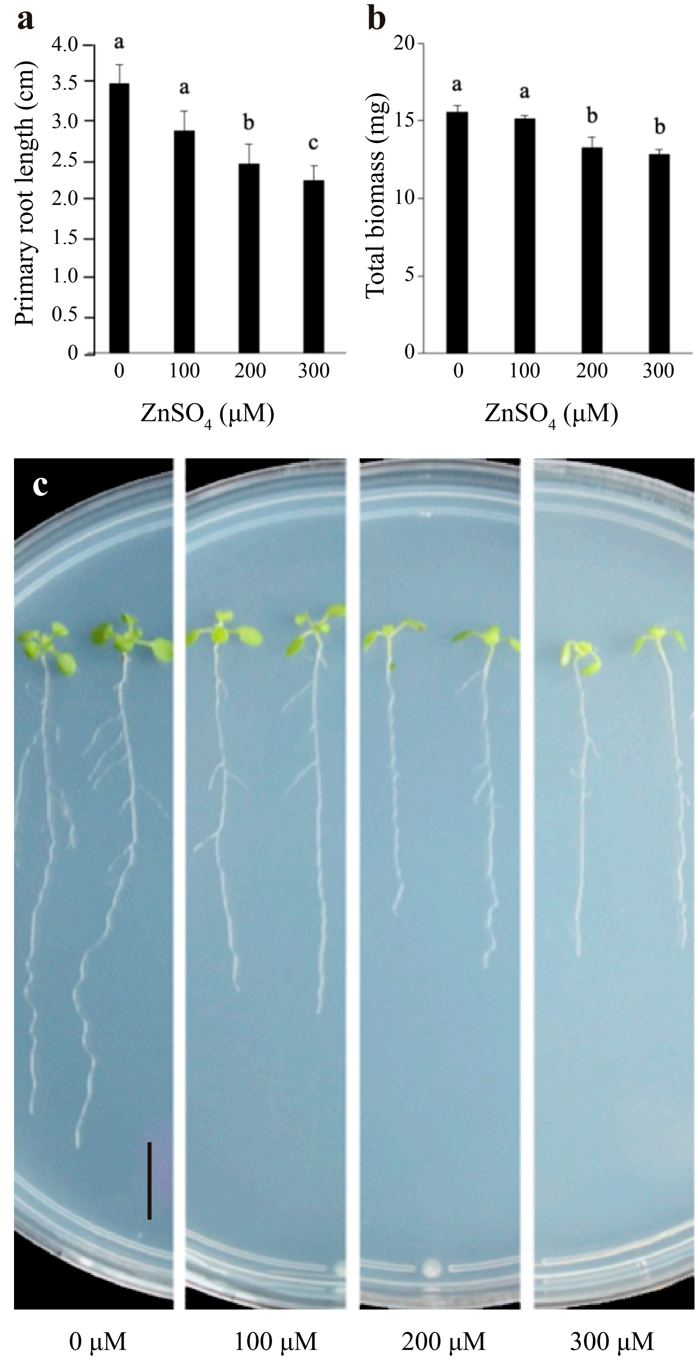

Figure 3. Effects of $ZnSO_4$ on *A. thaliana* growth. (**a**) Primary root length (cm). (**b**) Total biomass (mg). (**c**) Representative images of seedlings grown under increasing $ZnSO_4$ concentrations (0–300 µM) for 6 days post-transfer (6 DPT). Data represent mean ± SE (N = 18). Different letters indicate statistically significant differences among treatments (one-way ANOVA followed by Tukey's test; $p \leq 0.05$). Scale bar = 1 cm.

the bacterium. Under these conditions, bacterial volatile compounds did not significantly modify the effects of Zn on total biomass or primary root growth of *A. thaliana* (**Figure 4a, b**). However, using a cellular damage reporter line (*pERF115::GUS:GFP*), we found that exposure to 300 µM $ZnSO_4$ caused cellular damage in the primary root of *A. thaliana*. Notably, volatile compounds produced by *A. vinelandii* AEIV mitigated the cellular damage induced by exposure to this heavy metal (**Figure 4c**).

ABA is a phytohormone involved in plant responses to Zn

Arabidopsis reporter lines for different plant growth regulators were used to identify signaling pathways involved in *A. thaliana* responses to Zn exposure. Among the lines tested, *pABI5::GUS* showed clear changes in expression pattern and intensity in response to Zn (**Figure 5a**), indicating the involvement of ABA signaling in the response to this heavy metal. This result was not entirely unexpected, since ABA is a key phytohormone involved in plant responses to abiotic stress, such as heavy metal exposure (Hu *et al.,* 2022). Specifically, the reporter line revealed strong activation of the *ABI5* gene promoter in the roots of Zn-exposed seedlings. *ABI5* encodes a transcription factor regulating ABA-responsive genes involved in seed germination and abiotic stress responses (Skubacz *et al.,* 2016). Notably, promoter activity at 300 µM $ZnSO_4$ reached levels comparable to those induced by 15 µM ABA (**Figure 5a**).

Based on the results obtained with the *pABI5::GUS* reporter line, we next evaluated the direct involvement of ABI5 in response to Zn using the ABA-insensitive mutant *abi5*, which displays a longer primary root than its wild-type counterpart (Wassilewskija/Ws) (**Figure 5b**). Interestingly, the growth of this mutant, evaluated by the percentage of total biomass accumulation (**Figure 5c**), was less sensitive to Zn exposure, further supporting

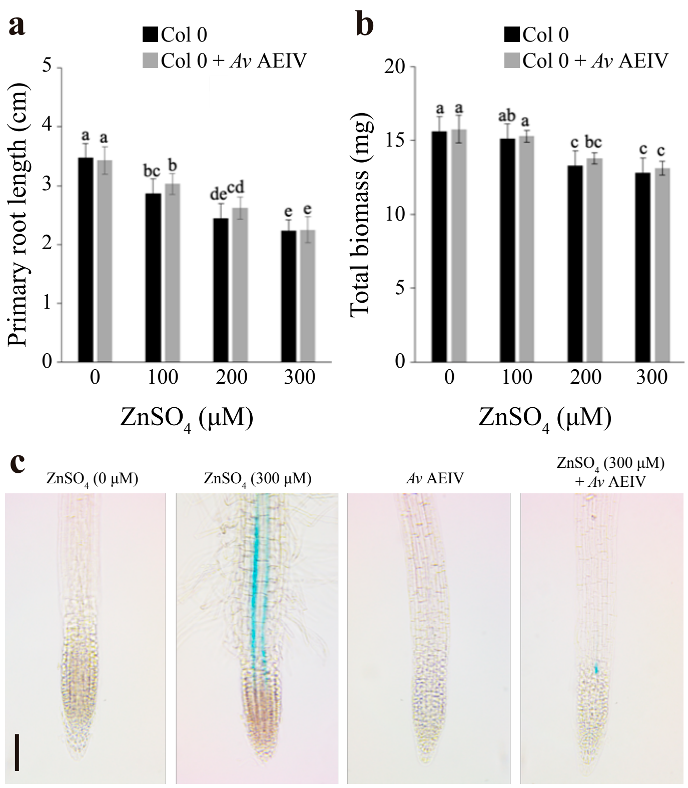

Figure 4. Effects of *A. vinelandii* AEIV volatiles on *A. thaliana* growth and cellular damage under Zn stress. (**a**) Primary root length (cm). (**b**) Total biomass (mg) in seedlings grown under increasing $ZnSO_4$ concentrations (0–300 µM) in the presence or absence of *A. vinelandii* AEIV volatiles (CoVo system). Data represent mean ± SE (N = 18). Different letters indicate statistically significant differences among treatments (one-way ANOVA followed by Tukey's test; $p \leq 0.05$). (c) Histochemical detection of cellular damage using the *pERF115::GUS:GFP* reporter line in response to 300 µM $ZnSO_4$ in the presence or absence of bacterial volatiles. Images were acquired at 20× magnification (scale bar = 100 µm). N = 12.

the involvement of the ABA signaling pathway, and specifically ABI5, in plant responses to this heavy metal.

Volatiles produced by *A. vinelandii* AEIV affect the ABA signaling pathway

We next used the *pABI5::GUS* reporter line to assess the effects of $ZnSO_4$ in the CoVo interaction system, in which the volatile compounds produced by *A. vinelandii* AEIV may influence ABA signaling. The results shown in **Figure 6** reveal that under Zn treatment, bacterial volatile compounds promoted *ABI5* gene expression along the root tip. This result provides evidence that the effects of *A. vinelandii* AEIV volatiles on plant responses to Zn involve the ABA signaling pathway, in which ABI5 plays a central role.

Discussion

Plant growth-promoting bacteria (PGPB) influence plant development through multiple mechanisms, both directly and indirectly, including enhanced nutrient availability, phytohormone production, synthesis of antimicrobial compounds and emission of inorganic/organic volatile compounds that reinforce plant immune responses (Mondal *et al.,* 2025; Srikamwang *et al.,* 2023). Another key aspect of PGPB is the modulation of root architecture, such as increasing root biomass through the formation of lateral roots and/or root hairs, thereby improving soil exploration and water and nutrient acquisition. Additionally, PGPB can promote shoot development (i.e., more and larger leaves), allowing increased photosynthetic area (Mantuano *et al.,* 2021; Liu *et al.,* 2023).

Azotobacter is a genus of free-living Gram-negative bacteria that has long been used as a biofertilizer due to its nitrogen fixation capacity, converting atmospheric N gas into ammonia (NH_3) available for plant uptake (Sumbul *et al*., 2020; Barney, 2024). Species such as *A. vinelandii* also produce phytohormones (auxins, gibberellins, cytokinins) and biopolymers such as alginate (extracellular) and poly-β-hydroxybutyrate (PHB; intracellular), which contribute to plant growth promotion. Notably, alginates contain negatively charged functional groups capable of adsorbing positively charged metal ions, including Zn and other heavy metals, thereby reducing their bioavailability and toxicity under adverse conditions (Burciaga-Montemayor *et al.,* 2020).

Zinc is an essential micronutrient for plants, acting as a cofactor for various enzymes involved in key metabolic processes such as photosynthesis, plant hormone regulation, nucleic acid synthesis, and fruit/seed production/maturation. Indeed, a computational analysis in *A. thaliana* identified over 1,000 proteins that bind, transport, or require Zn. However, as is the case with all heavy metals, excess Zn becomes toxic and negatively affects plant growth. In fact, Zn concentrations of 300 µM in the culture medium repress *A. thaliana* seed germination, induce leaf chlorosis, restrict root growth, and promote abnormal root hair formation (Fukao *et al.,* 2011; Assunção, 2022). In addition, excessive Zn in soils can disrupt the uptake of other essential nutrients, such as phosphorus, magnesium, and manganese, due to competitive interactions at the root level (Amezcua y Lara, 2017; Kaur y Garg, 2021).

Under heavy metal stress, plants activate multiple adaptive responses including, (1) enhancement of antioxidant system, (2) accumulation of osmoregulatory compounds, (3) modulation of photochemical systems, (4)

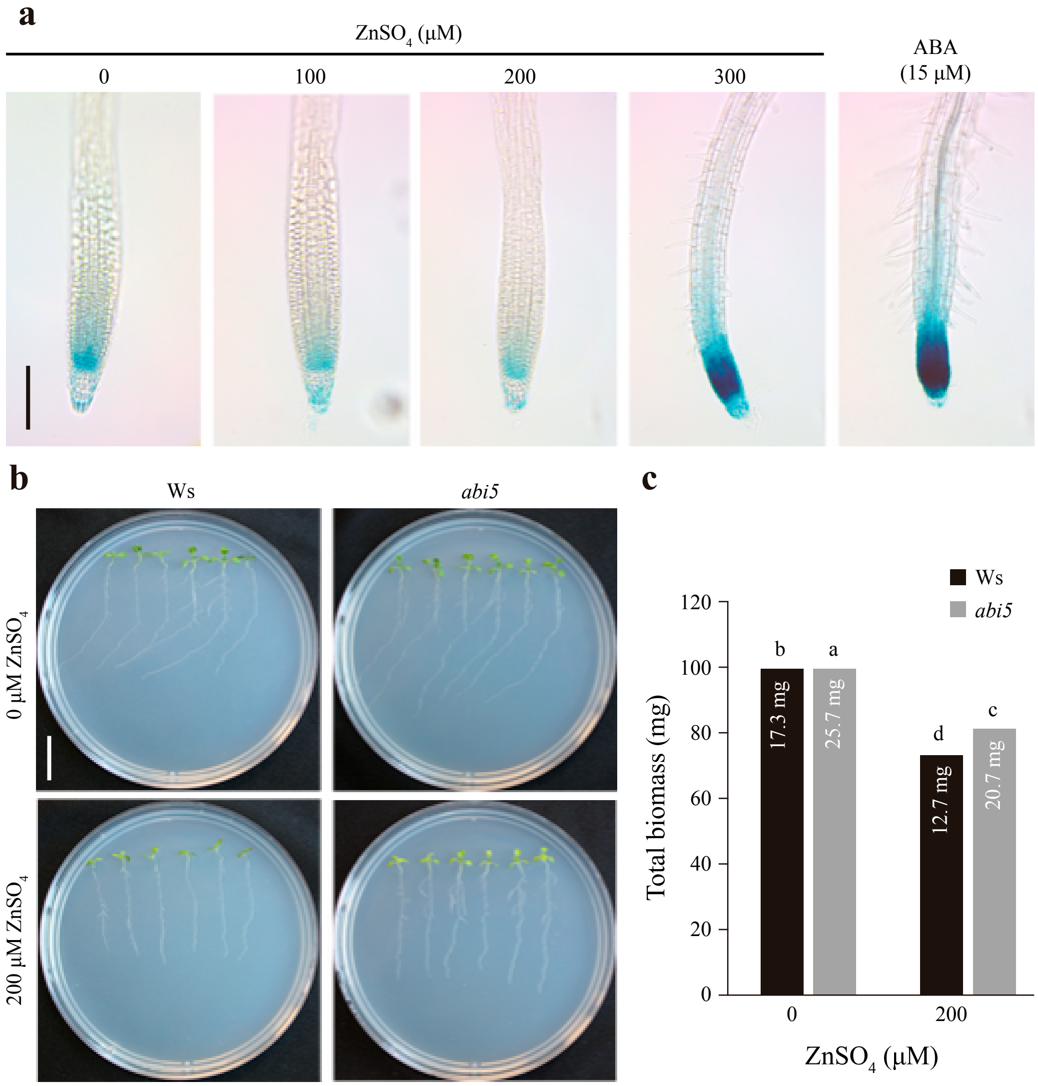

Figure 5. Involvement of ABA signaling in *A. thaliana* responses to Zn stress. (**a**) Expression of the *pABI5::GUS* reporter in root tips of *A. thaliana* seedlings exposed to $ZnSO_4$. Images were acquired at 20× magnification (scale bar = 100 μm). N = 12. (**b**) Representative phenotype of wild-type (Ws) and *abi5* mutant seedlings grown under control conditions and in the presence of 200 μM $ZnSO_4$. Scale bar = 1 cm. (**c**) Total biomass (% of control) of wild-type (Ws) and *abi5* mutant seedlings under control conditions and Zn treatment (200 μM). Data represent mean ± SE (N = 18). Average values for each treatment are shown within the bars, and the letters above the bars indicate statistically significant differences between them (one-way ANOVA followed by Tukey's test; $p \leq 0.05$).

restriction of metal uptake and translocation, (5) regulation of endogenous hormone levels, (6) reprogramming of gene expression, and (7) adjustment of plant-microorganism interactions (Feng *et al.,* 2023). Therefore, understanding how plants respond to high Zn concentrations at different stages of plant development is essential for implementing strategies to mitigate its harmful effects.

In this study, we established a plant-bacteria-heavy metal interaction system (*A. thaliana*-*A. vinelandii*-Zn) to evaluate the potential contribution of a free-living nitrogen-fixing PGPB in enhancing plant tolerance to Zn stress. The results showed that in two of the three interaction systems implemented (direct contact and diffusible plus volatile compounds), between *A. thaliana*-*A. vinelandii* has a negative effect on plant growth. This unexpected outcome is likely associated with the high bacterial inoculum, which may have led to strong competition for nutrients in the rhizosphere and/or exacerbated stress-related signaling due to the combined presence of bacteria and Zn. It is also important to note that since the Zn concentrations used in this study negatively affected the growth of both *A. thaliana* and *A. vinelandii*, the contribution of diffusible compounds produced by the bacteria in response to Zn stress that affect plant growth cannot be excluded.

In contrast, when only volatile-mediated interaction was allowed, *A. vinelandii* AEIV exerted a positive ef-

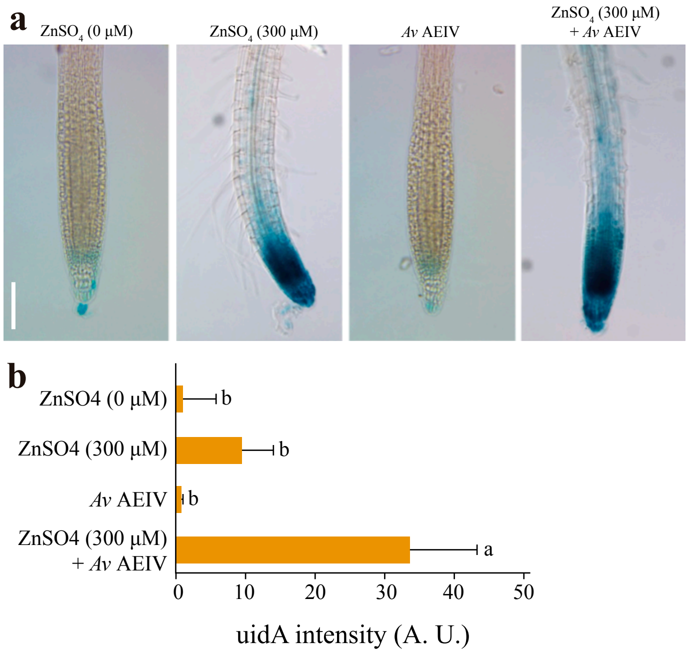

Figure 6. Effects of *A. vinelandii* AEIV volatiles on ABA signaling in *A. thaliana* under Zn stress. (**a**) Expression of the *pABI5::GUS* reporter in root tips of *A. thaliana* seedlings exposed to $ZnSO_4$ (0 or 300 µM) in the presence or absence of *A. vinelandii* AEIV volatiles (CoVo system). Images were acquired at 20× magnification (scale bar = 100 µm). N = 12. (**b**) Quantification of reporter activity (arbitrary units, A.U.) based on the images shown in (a). Data represent mean ± SE (N = 3 for controls and N = 6 for treatments). Different letters indicate statistically significant differences among treatments (one-way ANOVA followed by Tukey's test; $p \leq 0.05$).

fect on certain plant growth parameters. This observation led us to focus on this system to further investigate plant responses to Zn in the presence and absence of bacterial volatiles. Microorganisms are known to produce volatile compounds as products of their primary and secondary metabolism, and these compounds can stimulate plant growth, induce systemic resistance, or inhibit microbial competitors (Chandrasekaran *et al.,* 2022). In terms of plant health, volatiles produced by PGPB participate in plant-microorganism communication, modulating metabolic pathways and regulating the plant's defense responses (Rani *et al.,* 2023).

Although *A. vinelandii* AEIV volatiles did not significantly alleviate the negative effects of Zn on overall *A. thaliana* growth, they did mitigate Zn-induced cellular damage in root tissues. This suggests that their primary role may not be direct growth promotion under stress, but rather modulation of stress responses. The cellular damage most frequently associated with heavy metal stress includes excessive ROS accumulation, lipid peroxidation of membranes, loss of membrane integrity, and alterations of the photosynthetic apparatus. Several mechanisms, including preservation of membrane integrity, maintenance of photosynthetic activity, and modification of ROS availability/accumulation through increased expression/activity of antioxidant enzymes (SOD, APX, CAT), have been proposed regarding the potential contribution of PGPB to mitigating these effects (Ajmal *et al.*, 2022; Wang *et al.*, 2022). The mechanism by which *A. vinelandii* AEIV helps plants to mitigate zinc-induced cellular damage remains to be demonstrated.

In this context, our results indicate that Zn exposure activates an ABA-mediated signaling pathway, which is further influenced by bacterial volatiles. Specifically, the induction of *ABI5* gene expression, which encodes a transcription factor involved in ABA signaling, was observed under Zn stress. Since ABI5 is known to regulate the expression of genes associated with senescence (*SGR1, NYC1*) and energy storage (i.e., *DGAT1*), its overexpression could be related to growth limitation in response to Zn exposure (Hu *et al.,* 2022; Skubacz *et al.,* 2016).

To further support this proposal, the *abi5* mutant was included in the analysis of the Zn response. The reduced sensitivity of the *abi5* mutant to Zn further supports the central role of this transcription factor in mediating plant responses to heavy metal stress. Moreover, the enhanced expression of *ABI5* in the presence of *A. vinelandii* AEIV volatiles suggests that the bacterium modulates the *A. thaliana* ABA signaling pathway to cope with Zn exposure. These findings indicate that bacterial volatiles may function primarily as regulators of plant stress signaling rather than as direct protective agents against Zn toxicity.

Collectively, our results reveal the complexity of plant-microorganism-metal interactions and suggest that *A. vinelandii* AEIV contributes to plant adaptation to Zn stress through modulation of hormonal signaling pathways. In this context, ABI5 emerges as a key negative regulator of growth under Zn stress, and its manipulation could represent a potential biotechnological strategy to improve plant tolerance to heavy metals.

Conclusion

The results of this research provide evidence that plant tolerance to heavy metals such as Zn is a complex trait that depends on the nature of plant-microorganism interaction, as well as on hormonal signaling and associated changes in gene expression. In this scenario, modulation of genes such as *ABI5,* together with the use of volatile-producing PGPBs represent a promising strategy for developing crops with improved tolerance to polluted environments.

Acknowledgements

This work was supported by grants from the Universidad Nacional Autónoma de México (DGAPA-PAPIIT, Project: IN-203226), and the Secretaría de Ciencia, Humanidades, Tecnología e Innovación (SECIHTI Project: CF-2023-I-1605). PPL received an undergraduate scholarship from DGAPA-PAPIIT: IN 213723-095024 and KABR received a postdoctoral fellowship from SECIHTI: EPM I12090/311/2023.

References

Aasfar A, Bargaz A, Yaakoubi K, Hilali A, Bennis I, Zeroual Y, Meftah Kadmiri I (2021). Nitrogen fixing *Azotobacter* species as potential soil biological enhancers for crop nutrition and yield stability (2021). *Front Microbiol* 12:628379. doi:10.3389/fmicb.2021.628379

Ajmal AW, Yasmin H, Hassan MN, Khan N, Jan BL, Mumtaz S (2022). Heavy metal–resistant plant growth–promoting *Citrobacter werkmanii* strain WWN1 and *Enterobacter cloacae* strain JWM6 enhance wheat (*Triticum aestivum* L.) growth by modulating physiological attributes and some key antioxidants under multi-metal stress. *Front Microbiol*, 13:815704. doi:10.3389/fmicb.2022.815704

Amezcua J, Lara M (2017). El Zn en las plantas. *Ciencia* 68(3):28-35.

Assunção AGL (2022). The F-bZIP-regulated Zn deficiency response in land plants. *Planta* 256(6):108. doi:10.1007/s00425-022-04019-6

Barney BM. (2024). *Azotobacter vinelandii*. *Trends Microbiol* 32(10):1034-1035. doi:10.1016/j.tim.2024.07.006

Bazihizina N, Taiti C, Marti L, Rodrigo-Moreno A, Spinelli F, Giordano C, Caparrotta S, Gori M, Azzarello E, Mancuso S (2014). Zn^{2+} -induced changes at the root level account for the increased tolerance of acclimated tobacco plants. *J Exp Bot* 65:4931-4942. doi:10.1093/jxb/eru251

Burciaga-Montemayor NG, Claudio-Rizo JA, Cano-Salazar LF, Martínez-Luévanos A, Vega-Sánchez P (2020). Compósitos en estado hidrogel con aplicación en la adsorción de metales pesados presentes en aguas residuales. *TIP. Revista Especializada en Ciencias Químico-Biológicas* 23. doi:10.22201/fesz.23958723e.2020.0.211

Chandrasekaran M, Paramasivan M, Sahayarayan JJ (2022). Microbial volatile organic compounds: an alternative for chemical fertilizers in sustainable agriculture development. *Microorganisms* 11(1):42. doi:10.3390/microorganisms11010042

Das HK (2019). *Azotobacter* as biofertilizer. *Adv Appl Microbiol*, 108:1-43. doi:10.1016/bs.aambs.2019.07.001

Felemban A, Braguy J, Zurbriggen MD, Al-Babili S (2019). Apocarotenoids involved in plant development and stress response. *Front Plant Sci* 10:1168. doi:10.3389/fpls.2019.01168

Feng D, Wang R, Sun X, Liu L, Liu P, Tang J, Zhang C, Liu H (2023). Heavy metal stress in plants: Ways to alleviate with exogenous substances. *Sci Tot Environ* 897:165397. doi:10.1016/j.scitotenv.2023.165397

Ferjani A, Tsukagoshi H, Vassileva V (2023). Editorial: Model organisms in plant science: *Arabidopsis thaliana*. *Front Plant Sci* 14:1279230. doi:10.3389/fpls.2023.1279230

Finkelstein R (2013). Abscisic acid synthesis and response. *The Arabidopsis Book* 11:e0166. doi:10.1199/tab.0166

Fukao Y, Ferjani A, Tomioka R, Nagasaki N, Kurata R, Nishimori Y, Fujiwara M, Maeshima M (2011). iTRAQ Analysis reveals mechanisms of growth defects due to excess zinc in Arabidopsis. *Plant Physiol* 155(4):1893-1907. doi:10.1104/pp.110.169730

Gong Z, Xiong L, Shi H, Yang S, Herrera-Estrella LR, Xu G, Chao DY, Li J, Wang PY, Qin F, Li J, Ding Y, Shi Y, Wang Y, Yang Y, Guo Y, Zhu JK (2020). Plant abiotic stress response and nutrient use efficiency. *Sci China Life Sci* 63:635-674. doi:10.1007/s11427-020-1683-x

Hasan A, Tabassum B, Hashim M, Khan N (2024). Role of plant growth promoting rhizobacteria (PGPR) as a plant growth enhancer for sustainable agriculture: A Review. *Bacteria* 3(2):59-75. doi:10.3390/bacteria3020005

Heyman J, Cools T, Canher B, Shavialenka S, Traas J, Vercauteren I, Van den Daele H, Persiau G, De Jaeger G, Sugimoto K, De Veylder L (2014). The heterodimeric transcription factor complex ERF115-PAT1 grants regeneration competence. *Nat Plants* 2(11):16165. doi:10.1038/nplants.2016.165

Hu W, Zhang J, Wu Z, Loka DA, Zhao W, Chen B, Wang Y, Meng Y, Zhou, Z, Gao L (2022). Effects of single and combined exogenous application of abscisic acid and melatonin on cotton carbohydrate metabolism and yield under drought stress. *Ind Crops Prod* 176:114302. doi:10.1016/j.indcrop.2021.114302

Kaur H, Garg N (2021). Zinc toxicity in plants: a review. *Planta* 253(6):129. doi:10.1007/s00425-021-03642-z

Larsen B, Haug A (1971). Biosynthesis of alginate. 1. Composition and structure of alginate produced by *Azotobacter vinelandii* (Lipman). *Carbohydr Res* 17(2):287-296. doi:10.1016/s0008-6215(00)82536-7

Lim J, Lim CW, Lee SC (2022). Core components of abscisic acid signaling and their post-translational modification. *Front Plant Sci* 13:895698. doi:10.3389/fpls.2022.895698

Liu D, Mou S, Zou Y, Yang B, Ding R, Nie J, Zhang X, Jia Z, Han Q (2023). Exploring the relationship between deep roots and shoot growth of wheat under different soil moisture: A large soil column experiment 1. *Rhizosphere* 25:100675. doi:10.1016/j.rhisph.2023.100675

López-Molina L, Mongrand S, Chua NH (2001). A post-germination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in Arabidopsis. *PNAS USA* 98:4782-4787. doi:10.1073/pnas.081594298

Mantuano D, Ornellas T, Aidar MPM, Mantovani A (2021). Photosynthetic activity increases with leaf size and intercellular spaces in an allomorphic lianescent aroid *Rhodospatha oblongata. Funct Plant Biol* 48:557-566. doi:10.1071/FP20215

Miki B, McHugh S (2004). Selectable marker genes in transgenic plants: applications, alternatives and biosafety. *J Biotechnol* 107(3):193-232. doi:10.1016/j.jbiotec.2003.10.011

Mondal S, Sarkar O, Mandal SM, Chattopadhyay A, Sahoo P (2025). Monitoring CO as a plant signaling molecule under heavy metal stress using carbon nanodots. *Dalton Trans* 54(20):8214-8223. doi:10.1039/d4dt03101f

Nakayama S, Sugano SS, Hirokawa H, Mori IC, Daimon H, Kimura S, Fukao Y (2020). Manganese treatment alleviates Zn deficiency symptoms in *Arabidopsis* seedlings. *Plant, Cell Physiol* 61:1711-1723. doi:10.1093/pcp/pcaa094

Ng LM, Melcher K, Teh BT, Xu HE (2014). Abscisic acid perception and signaling: structural mechanisms and applications. *Acta Pharmacol Sin* 35:567-584. doi:10.1038/aps.2014.5

Núñez C, Peña C, Kloeckner W, Hernández-Eligio A, Bogachev AV, Moreno S, Guzmán J, Büchs J, Espín G (2013). Alginate synthesis in *Azotobacter vinelandii* is increased by reducing the intracellular production of ubiquinone. *Appl Microbiol Biotechnol* 97(6):2503-2512. doi:110.1007/s00253-012-4329-0

Pantigoso HA, Newberger D, Vivanco JM (2022). The rhizosphere microbiome: Plant-microbial interactions for resource acquisition. *J Appl Microbiol* 133:2864-2876. doi:10.1111/jam.15686

Rani A, Rana A, Dhaka RK, Singh AP, Chahar M, Singh S, Nain L, Singh KP, Minz D (2023). Bacterial volatile organic compounds as biopesticides, growth promoters and plant-defense elicitors: current understanding and future scope. *Biotech Adv* 63:108078. doi:10.1016/j.biotechadv.2022.108078

Sahoo RK, Rani V, Tuteja N (2021). *Azotobacter vinelandii* helps to combat chromium stress in rice by maintaining antioxidant machinery. *Biotech* 11(6):275. doi:10.1007/s13205-021-02835-3

Shaffique S, Hussain S, Kang SM, Imran M, Kwon EH, Khan MA, Khan MA, Lee IJ (2023). Recent progress on the microbial mitigation of heavy metal stress in soybean: overview and implications. *Front Plant Sci* 14:1188856. doi:10.3389/fpls.2023.1188856

Skubacz A, Daszkowska-Golec A, Szarejko I (2016). The role and regulation of ABI5 (ABA-Insensitive 5) in plant development, abiotic stress responses and phytohormone crosstalk. *Front Plant Sci* 7:1884. doi:10.3389/fpls.2016.01884

Srikamwang C, Onsa NE, Sunanta P, Sangta J, Chanway CP, Thanakkasaranee S, Sommano SR (2023). Role of microbial volatile organic compounds in promoting plant growth and disease resistance in horticultural production. *Plant Signal Behav* 18(1):2227440. doi:10.1080/15592324.2023.2227440

Stuiver CEE, Posthumus FS, Parmar S, Shahbaz M, Hawkesford MJ, De Kok LJ (2014). Zn exposure has differential effects on uptake and metabolism of sulfur and nitrogen in Chinese cabbage. *J Plant Nut Soil Sci* 177:748-757. doi:10.1002/jpln.201300369

Sumbul A, Ansari RA, Rizvi R, Mahmood I (2020). *Azotobacter*: A potential bio-fertilizer for soil and plant health management. *Saudi J Biol Sci* 27(12):3634-3640. doi:10.1016/j.sjbs.2020.08.004

Wang Y, Narayanan M, Shi X, Chen X, Li Z, Natarajan D, Ma Y (2022). Plant growth-promoting bacteria in metal-contaminated soil: current perspectives on remediation mechanisms. *Front Microbiol* 13:966226. doi:10.3389/fmicb.2022.966226

Yoshida N, Takase R, Sugahara Y, Nambu Y, Hashimoto W (2022). Direct production of polyhydroxybutyrate and alginate from crude glycerol by *Azotobacter vinelandii* using atmospheric nitrogen. *Sci Rep* 12(1):8032. doi:10.1038/s41598-022-11728-1