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Environmental pollutants in agroecosystem: Toxicity, mechanism, and remediation

Edited by
Muhammad Musa Khan and Pankaj Bhatt

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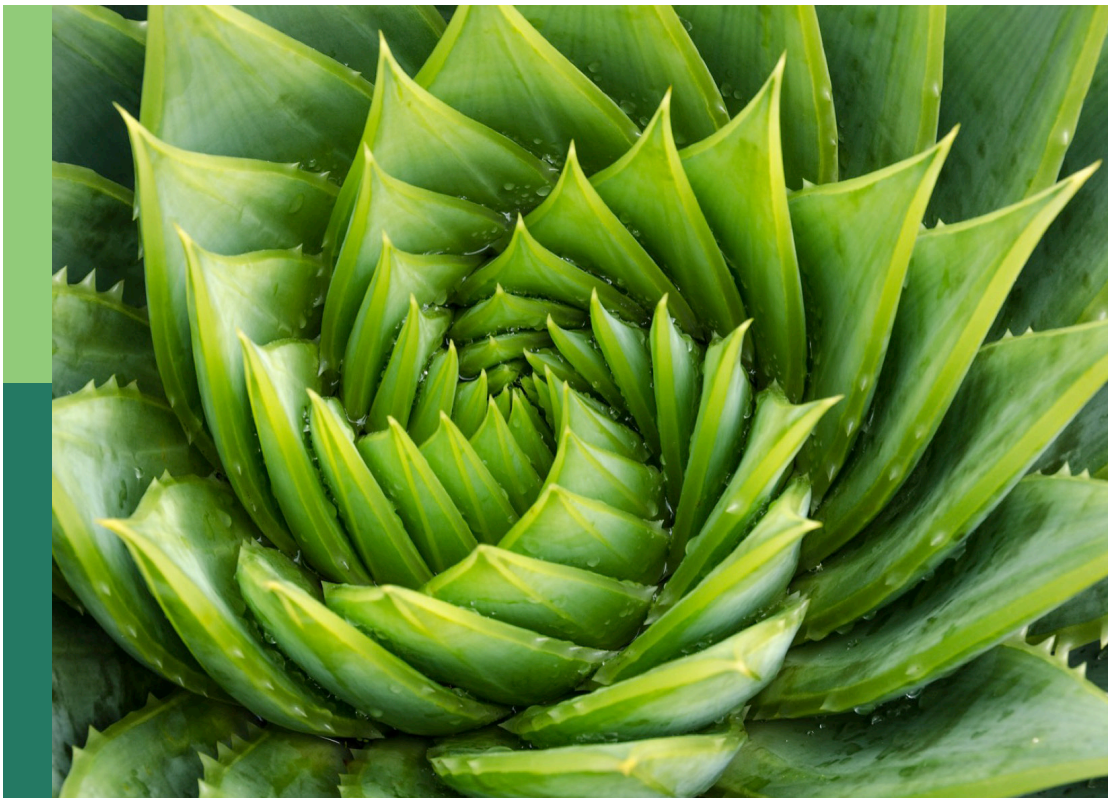

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Environmental pollutants in agroecosystem: Toxicity, mechanism, and remediation

Topic editors

Muhammad Musa Khan — Zhejiang University, China

Pankaj Bhatt — Purdue University, United States

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EDITED BY
Fernanda Fidalgo,
University of Porto, Portugal

REVIEWED BY
Ricardo Azevedo,
Universidade de São Paulo, Brazil

*CORRESPONDENCE
Muhammad Musa Khan
drmusa@zju.edu.cn

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Editorial: Environmental pollutants in agroecosystem: toxicity, mechanism, and remediation

Muhammad Musa Khan[1]* and Pankaj Bhatt[2]

[1]Plant Inspection, Quarantine and Crop Health Team, Hainan Institute of Zhejiang University, Sanya, China, [2]Department of Agricultural & Biological Engineering, Purdue University, West Lafayette, IN, United States

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environmental pollution, plants, vertebrates, invertebrates, biological and physiological stress, heavy metals, pesticides, microplastics

Editorial on the Research Topic
Environmental pollutants in agroecosystem: toxicity, mechanism, and remediation

1 Introduction

Environmental contamination in agroecosystems is a significant problem caused by various pollutants that can impact soil, water, air, and the surrounding ecosystem. Agroecosystems are complex systems where agriculture, ecology, and the environment interact, and contamination in one aspect can have cascading effects on the entire system (Wang et al., 2020). Contaminants in agroecosystems can come from various sources, including agricultural practices, industrial activities, and urbanization (Singh and Sharma, 2021).

Some agroecosystems' most common environmental contaminants are heavy metals, pesticides, and emerging pollutants (Picó et al., 2020). Heavy metals such as cadmium, lead, and mercury among others can accumulate in the soil and crops, leading to potential health hazards (Zwolak et al., 2019). Pesticides, including herbicides, insecticides, and fungicides, can also contaminate soil, water, and air, adversely affecting non-target organisms (Li et al., 2023). Emerging pollutants, such as pharmaceuticals and personal care products, are a growing concern in agroecosystems, as they can persist in the environment and pose long-term risks to ecosystem health (Osuoha et al., 2023). As different environmental pollutants have attracted attention, potential remediation techniques and methods have developed rapidly (Sun et al., 2018).

In this editorial, we set up a Research Topic of *Environmental pollutants in an agroecosystem: Toxicity, Mechanism, and Remediation*, which covers not only environmental pollutants in agroecosystems but also in the aquatic environment. The following themes are included in this Research Topic: (a) Risk assessment of environmental pollutants to plants; (b) Biological alterations induced by pollutants in plants and invertebrates; (c) Physiological and molecular mechanism of plants and vertebrates against pollutants; (d) Environmental

pollutants as a risk to agricultural practices; (e) Remediation techniques for environmental pollutants in the field.

Despite significant advances in understanding the environmental consequences of environmental pollutants, there remain knowledge gaps in these areas, and our Research Topic aims to address these gaps. In the end, we accepted and published 10 articles written by 99 researchers from seven different countries, such as China, India, Saudi Arabia, Pakistan, South Korea, Portugal, and the United Kingdom.

2 Remediation of the toxic pollutants

Remediation of toxic pollutants is an important process to protect human health and the environment by eliminating or mitigating the harmful effects of these substances. Various methods are available for remediating toxic pollutants, depending on the type and severity of contamination. Here are some common techniques which were reported in this research Topic:

- Bioremediation: this technique uses living organisms, such as bacteria, fungi, and plants, to break down, remove, or neutralize contaminants in soil, water, and air. The organisms may be naturally occurring or genetically engineered to enhance their remediation capabilities.
- Chemical treatment: chemicals react with pollutants, neutralizing or transforming them into less harmful substances. Examples include oxidation, reduction, precipitation, and neutralization processes.
- Phytoremediation: this technique uses plants to absorb, accumulate, or break down pollutants in the soil, water, or air. Some plants, such as sunflowers, are particularly effective at extracting heavy metals and other contaminants.

-Zhao et al. reported that foliar silicon (Si) spraying could reduce rice's cadmium (Cd) contamination, but different rice varieties respond differently. Si-inhibited varieties show a decrease in Cd content by 13.5%–65.7%, while Si-stimulated varieties experience an increase of 15.7%–24.1%. This highlights the importance of considering rice variety differences when implementing foliar Si spraying to remediate Cd-contaminated paddy fields.

-Lei et al. reported silicon's role in mitigating cadmium toxicity in plants. Silicon helps reduce cadmium uptake and transport, improves nutrient supply, regulates antioxidant systems, and enhances physical plant structure. The review specifically focuses on silicon's role in maintaining water balance and suggests future research directions.

-Huang et al. duckweed have phytoremediation ability and stated that high streptomycin concentrations negatively impact duckweed health, reducing biomass and growth rate while increasing antioxidant enzymes. However, duckweed demonstrates a high ability to remove streptomycin from the environment, with significant reductions observed after 20 days. This suggests that duckweed could be a valuable resource for treating aquaculture wastewater and domestic sewage contaminated with streptomycin.

-Zhu et al. stated that antioxidant enzymes and non-enzymatic antioxidants increased with Pb concentration, while peroxidase and the ascorbic acid-glutathione cycle showed mixed results. Through transcriptome sequencing, 17 root Pb-tolerant genes were identified, associated with antioxidant, transport, and transcription functions.

-Hafeez et al. reported that different plant hosts affected the bio-detoxification of insects differently by affecting the gene expression of a specific gene. This not only affects the efficiency of an insecticide against a pest but could also contribute to the development of insecticide resistance. The results suggested that the P450 enzyme system helps the herbivores adapt to the diverse host plant by developing different secondary compounds in their hosts.

-The study presented by Ejaz et al. focuses on the mechanism of heavy metals uptake in plant and their detoxification. The results stated that heavy metal concentrations exceeding permissible limits seriously threaten humans, plants, and other life forms. Plants absorb these toxic metals and employ strategies to cope with the contamination, such as restricting heavy metals within cell walls or synthesizing compounds to bind metal ions. They highlighted the importance of studying model plant species' genetics, molecular, and cell signaling aspects to understand their heavy metal tolerance strategies and potentially apply that knowledge to mitigate the negative effects of heavy metal contamination.

3 Rhizo-microbiome in environmental sustainability

The rhizo-microbiome, also known as the rhizosphere microbiome, is a complex community of microorganisms that resides in the soil surrounding plants roots i.e. rhizosphere. This diverse group of microbes, including bacteria, fungi, and archaea, is vital in promoting plant health, growth, and overall ecosystem functioning. Understanding and harnessing the potential of the rhizo-microbiome can greatly contribute to environmental sustainability. Some of how the rhizo-microbiome supports sustainability include:

- Nutrient cycling: rhizo-microbes play a crucial role in nutrient cycling, including nitrogen fixation, phosphorus solubilization, and potassium release. By converting these nutrients into plant-available forms, the rhizo-microbiome helps reduce the need for chemical fertilizers, which can have negative environmental impacts.
- Soil health and structure: rhizo-microbes contribute to soil aggregation and improve soil structure, which can prevent soil erosion, promote water infiltration, and enhance overall soil health. Healthy soils are essential for sustainable agriculture and long-term ecosystem stability.
- Phytoremediation: some plants, in association with their rhizo-microbes, can uptake and degrade pollutants from the soil, known as phytoremediation. This can help restore contaminated lands and improve overall environmental quality.

Rhizo-microbiome plays a vital role in maintaining and enhancing environmental sustainability. By understanding and harnessing the potential of these microbial communities, we can develop more sustainable agricultural practices, protect and restore ecosystems, and mitigate the impacts of climate change.

-The research conducted by Patani et al. reported that out of 107 PGPR strains, five *Bacillus* strains significantly improve tomato plant growth and productivity. Inoculated salt-stressed tomato plants had higher levels of essential nutrients and antioxidant enzyme activity while maintaining lower levels of harmful ions, indicating that halotolerant PGPR strains can mitigate the negative effects of salt stress on tomato plants.

-Li et al. explained how *Dahlia pinnata* accumulate Cd and detoxify heavy metal and the role of rhizospheric microbiota in phytoremediation.

4 Future research

In summary, the articles published in this Research Topics suggest that the research conducted thus far in these topics has significantly improved our comprehension of the environmental fate, ecotoxicology, risk assessment, and remediation of various pollutants. However, considerable challenges persist in the realm of computational toxicology, particularly in predicting the environmental risks posed by pollutants and understanding the combined effects of various contaminants (such as heavy metals, PPCPs, and microplastics). Further investigation is necessary in these domains, with a particular emphasis on the transfer of pollutants across different trophic levels, their biodegradation, and the mechanisms behind their impact.

Author contributions

MK authored the initial draft of this editorial, which PB subsequently revised and approved for submission. As guest topic editors, both MK and PB have been heavily involved in the call for submissions and have overseen the editing process for the manuscripts submitted to this Research Topic.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Pankaj Bhatt,
Purdue University, United States

REVIEWED BY
Hai-Ming Zhao,
Jinan University, China
Muhammad Rizwan Ul Haq,
King Faisal University, Saudi Arabia

*CORRESPONDENCE
Shaohua Chen
shchen@scau.edu.cn
Huishan Wang
huishanwang3@outlook.com
Lisheng Liao
lishengliao@scau.edu.cn

†These authors have contributed equally to this work

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A novel bacterial strain *Burkholderia* sp. F25 capable of degrading diffusible signal factor signal shows strong biocontrol potential

Hongxiao Yu[1†], Wen-Juan Chen[1,2†], Kalpana Bhatt[3], Zhe Zhou[1,2], Xixian Zhu[1,2], Siqi Liu[1], Jiehua He[1], Lian-Hui Zhang[1,2], Shaohua Chen[1,2*], Huishan Wang[1,2*] and Lisheng Liao[1,2*]

[1]Department of Plant Pathology, College of Plant Protection, South China Agricultural University, Guangzhou, China, [2]Guangdong Province Key Laboratory of Microbial Signals and Disease Control, Integrative Microbiology Research Centre, South China Agricultural University, Guangzhou, China, [3]Department of Botany and Microbiology, Gurukula Kangri University, Haridwar, Uttarakhand, India

Vast quantities of synthetic pesticides have been widely applied in various fields to kill plant pathogens, resulting in increased pathogen resistance and decreased effectiveness of such chemicals. In addition, the increased presence of pesticide residues affects living organisms and the environment largely on a global scale. To mitigate the impact of crop diseases more sustainably on plant health and productivity, there is a need for more safe and more eco-friendly strategies as compared to chemical prevention. Quorum sensing (QS) is an intercellular communication mechanism in a bacterial population, through which bacteria adjust their population density and behavior upon sensing the levels of signaling molecules in the environment. As an alternative, quorum quenching (QQ) is a promising new strategy for disease control, which interferes with QS by blocking intercellular communication between pathogenic bacteria to suppress the expression of disease-causing genes. Black rot caused by *Xanthomonas campestris* pv. *campestris* (*Xcc*) is associated with the diffusible signal factor (DSF). As detailed in this study, a new QQ strain F25, identified as *Burkholderia* sp., displayed a superior ability to completely degrade 2 mM of DSF within 72 h. The main intermediate product in the biodegradation of DSF was identified as n-decanoic acid, based on gas chromatography-mass spectrometry (GC-MS). A metabolic pathway for DSF by strain F25 is proposed, based on the chemical structure of DSF and its intermediates, demonstrating the possible degradation of DSF *via* oxidation-reduction. The application of strain F25 and its crude enzyme as biocontrol agents significantly attenuated black rot caused by *Xcc*, and inhibited tissue maceration in the host plant *Raphanus sativus* L., without affecting the host plant. This suggests that agents produced from strain F25 and its crude enzyme have promising applications in controlling infectious diseases

caused by DSF-dependent bacterial pathogens. These findings are expected to provide a new therapeutic strategy for controlling QS-mediated plant diseases.

KEYWORDS

diffusible signal factor (DSF), quorum quenching, quorum sensing, Burkholderia, biocontrol, plant diseases

Introduction

Quorum sensing (QS) is commonly observed in microorganisms regulating biofilm formation and cell growth (Hammond et al., 2015; Watve et al., 2020; Mishra et al., 2022). It also facilitates important biological functions such as extracellular product synthesis, bioluminescence, and the production of virulence factors (Semighini et al., 2006; Deng et al., 2014; Bukvicki et al., 2016). Population sensing is a communication mechanism between bacteria, referring to the process by which bacteria adjust their population density and behavior by sensing the levels of various signaling molecules in their environment (Ya'ar Bar et al., 2021; Liu et al., 2022; Zhou et al., 2022). Changes in the species composition and cell density of microbial communities can transmit information through complex signaling systems, allowing the bacteria to collectively change their behavior by exchanging information among themselves (Wang et al., 2004; Whiteley et al., 2017). The diffusible signal factor (DSF) family QS system is a conserved cellular communication system widely found in Gram-negative bacteria, involved in regulating the production of toxic substances by Gram-negative bacteria (Deng et al., 2016; Cui et al., 2018; Ye et al., 2019). The DSF family QS system has been confirmed to exist in a variety of *Xanthomonas* species (Bi et al., 2014; Kanugala et al., 2019), such as *Xanthomonas campestris* pv. *campestris* (*Xcc*) (Ryan et al., 2015), *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Chatterjee and Sonti, 2002), *Xanthomonas axonopodis* pv. *citri* (*Xac*) (Deng et al., 2011), and *Xanthomonas axonopodis* pv. *glycines* (*Xag*) (Park et al., 2019). Among them, *Xcc* causes cruciferous black rot, a plant disease that has a significant impact worldwide and has consequently attracted a lot of attention (Zhou et al., 2017; Rubel et al., 2019).

Black rot caused by *Xcc* occurs in vegetables in the cruciferous family such as radishes, cabbage, mustard, cauliflower, and kale (Mishra and Arora, 2012). The black rot pathogen usually multiplies and spreads rapidly in the field in warm and humid climates, causing black rot on the young stems and leaves of the plants, resulting in a severe reduction in crop quality and yield (An et al., 2020). In actual production, the control measures taken against the causative agent of black rot mainly include chemical control, such as chlorothalonil, mancozeb, agricultural streptomycin, among others (Molitor and Beyer, 2014; Sreelatha et al., 2022). It is well-known that the massive use of chemical pesticides causes serious environmental pollution, a series of safety problems (e.g., food safety), and affects human health (McEwen and Collignon, 2018; Ye et al., 2020c). Indiscriminate antibiotic use may lead to more pathogenic bacteria developing specific or even multi-drug resistance, hindering the control of diseases such as black rot (Agerso et al., 2007; Alsan et al., 2015; George, 2018). Therefore, it is urgent to develop environmentally friendly and efficient control strategies (Majumdar and Pal, 2016; Mukherjee and Bassler, 2019).

A series of extracellular enzymes produced by *Xcc* play an important role in the pathogenesis of the bacteria after the host plant is infected (Hsiao et al., 2010; Timilsina et al., 2020; Islam et al., 2021). The signaling molecule DSF has been identified as *cis*-11-methyl-2-dodecenoic acid and was associated with the pathogenic process caused by *Xcc* (He and Zhang, 2008; Deng et al., 2015; Zhou et al., 2015). Suppressing the expression of pathogenic genes during QS through blocking intercellular communication between pathogenic bacteria interferes with QS and has been recognized as a highly promising disease control measure, also known as quorum quenching (QQ) (Gu et al., 2018; Xu et al., 2021; Wang et al., 2022). QQ is a new strategy for disease control, proposed based on QS. It acts as a biological control by inhibiting the synthesis, accumulation, and monitoring of signaling molecules (Nhan et al., 2010). The quorum sensing system can also be interfered with through enzymatic degradation or modification of the signaling molecules (Zhang et al., 2019). In this way, the goal of inhibiting the expression of genes related to the pathogenicity of microorganisms can be achieved, thus attenuating their pathogenicity (Zhang, 2003). Ultimately, the purpose of disease control can be achieved. Degradation of microbial signaling molecules using quenching sterilization or quenching enzymes is, at present, the least toxic and most effective pathway for quorum quenching (Chen et al., 2013; Bhatt et al., 2022). QQ is carried out by regulating the QS system to control diseases and does not produce selection pressure on microorganisms, such that the pathogenic bacteria do not develop resistance (Steindler and Venturi, 2007). There are currently three methodological approaches to QQ: the first is based on quorum sensing inhibitors (QSIs), such as the inhibitor halogenated furanones first identified from marine red algae (*Deliseapulchra*)

(Wopperer et al., 2006), whose mechanism is to inhibit the synthesis of the signaling molecule (Kim et al., 2007); the second involves the use of a structural analogue of the signaling molecule, whose mechanism is to interfere with the binding of the signaling molecule to the receptor protein by competitively binding to the corresponding receptor protein (Murugayah and Gerth, 2019); and the third is a quenching molecule or quenching enzyme, whose mechanism is to degrade the signaling molecule such that it does not reach a certain threshold value (Hong et al., 2012; Rolland et al., 2016). Overall, quorum quenching or quenching enzymes acting outside the cell can avoid (or, at least, reduce) the selection pressure on cells, compared to inhibitors of signaling molecules. With more in-depth studies, QQ pathways based on quenching and sterilizing or quenching enzymes for plant disease control are expected to achieve significant breakthroughs that cannot be achieved by traditional chemical control means (Turan et al., 2017; Leguina et al., 2018; Wang et al., 2020).

Many Gram-negative bacteria rely on the QS system to detect their population density and activate the expression of some relevant genes by releasing an accumulation of signaling molecules (Zhang et al., 2020). There are two main approaches to quorum quenching sterilization with biocontrol effects. On one hand, quorum quenching enzyme genes can be transferred into microorganisms to obtain transgenic quenching sterilization (Dong and Zhang, 2005). On the other hand, quorum quenchers can be screened from nature, as many microbial taxa can degrade *N*-acyl homoserine lactone (AHL) signaling molecules (Huang et al., 2016; Wang et al., 2022). Recently, several microbial strains such as *Acinetobacter lactucae* QL-1 (Ye et al., 2019), *Pseudomonas* sp. HS-18 (Wang et al., 2020), and *Cupriavidus pinatubonensis* HN-2 (Xu et al., 2021) capable of degrading DSF have been isolated and characterized. However, genetically engineered quenchers have not yet been widely accepted, as they are not easy to cultivate. In contrast, there exist a large number of microorganisms in nature that can degrade signaling molecules, which are easy to cultivate in large numbers (Ye et al., 2020a; Ye et al., 2020b). Therefore, screening populations for quenching sterilization from nature present more obvious advantages for application as a biodegradation agent.

In this study, a strain of *Burkholderia* sp. F25 is identified as a significant degrader of DSF, and the degradation mechanism, degradation products, and degradation capacity of F25 are investigated. This bacterium can degrade DSF rapidly and efficiently, presents a quorum quenching function, and has a significant biological effect on the prevention of DSF-dependent diseases, such as black rot caused by *Xcc* XC1. In addition, the properties and biocontrol effects of crude enzymes extracted from F25 are investigated. This study, considering strain F25 as a biocontrol agent, provides new ideas to clarify the pathogenic regulatory mechanisms of DSF signaling-mediated pathogens, as well as new insights for the development of biocontrol strategies against DSF signaling-mediated bacterial pathogens.

Materials and methods

Chemicals and plants

Diffusible signal factor (DSF) (≥99%) was purchased from Shanghai UDChem Technology Co., Ltd (Shanghai, China) and dissolved in methanol to create a stock solution with a concentration of 100 mmol·L^{-1}. Radishes (*Raphanus sativus* L.) were purchased from a local market (Guangzhou, China) and healthy plants were selected for the biocontrol experiments.

Ampicillin (AMP, 50 mg·mL^{-1}), gentamicin (GEN, 50 mg·mL^{-1}), neomycin sulfate (NEO, 50 mg·mL^{-1}), carbenicillin (CARB, 50 mg·mL^{-1}), chloramphenicol (CM50, 30 mg·mL^{-1}), tetracycline (TC, 5 mg·mL^{-1}), kanamycin (KAN, 50 mg·mL^{-1}), and streptomycin (STR, 50 mg·mL$^{-1)}$, were used for antibiotic susceptibility testing, purchased from Sigma Aldrich Chemicals Co., Ltd (Shanghai, China).

Strains and culture conditions

XC1 was provided by the Integrative Microbiology Research Centre, South China Agricultural University, Guangzhou, China. *Xcc* were cultured on Luria–Bertani (LB) medium (NaCl 10.0 g·L^{-1}, tryptone 10.0 g·L^{-1}, and yeast extract 5.0 g·L^{-1}) with rifampicin (30 μg·mL^{-1}) at 28°C. The isolates were grown on LB medium or mineral salt medium (MSM: $(NH_4)_2SO_4$ 2.0 g·L^{-1}, $Na_2HPO_4{\cdot}12H_2O$ 1.5 g·L^{-1}, KH_2PO_4 1.5 g·L^{-1}, $MgSO_4{\cdot}7H_2O$ 0.2 g·L^{-1}, $CaCl_2{\cdot}2H_2O$ 0.01 g·L^{-1}, $FeSO_4{\cdot}7H_2O$ 0.001 g·L^{-1}; pH 7.2) with DSF (2 mmol·L^{-1}) at 30°C. The MSM medium and minimal medium (MM: $(NH_4)_2SO_4$, 2.0 g; $MgSO_4{\cdot}7H_2O$, 0.2 g; $CaCl_2$, 0.01 g; $FeSO_4$, 0.005 g; $MnCl_2$, 0.002 g; K_2HPO_4, 10.5 g; KH_2PO_4, 4.5 g; mannitol, 2.0 g; glycerol, 2.0 g; 1000 mL of H_2O; pH 6.5) were used to test the degradation efficiency of strain F25 on diffusible signal factor (DSF) and for the identification of metabolites.

Isolation and screening of *Burkholderia* sp. strain F25

Soil samples were collected on March 16, 2017, from the surface layer to a depth of 5 cm, at a perennially cultivated sweet potato field in Heshun Lugang, Nanhai District, Foshan City, Guangdong Province (Longitude: 113.14299°; Latitude: 23.02877°). The soil was sampled, bagged, and preserved as a microbial source for strain isolation. MSM medium was prepared, and 50 mL of MSM medium was sterilized in 250 mL triangular flasks. After cooling, DSF mother liquor (mother liquor concentration, 100 mM; methanol as solvent) was added under aseptic conditions, to make the final mass concentration of DSF 0.01 mM. Then, 5 g of soil sample was added, and the solution was incubated at 30°C in a 200 rpm shaker for 7 d. After this, it was transferred to the second batch of MSM medium, with a final mass concentration of DSF of

100 μM at 10% inoculum. After 7 d of incubation under the same conditions, the sample was transferred to MSM medium with a final mass concentration of 200 μM DSF at 10% inoculum, and incubated for another 7 d. The mass concentration of DSF was increased continuously. Then, 1 ml of MSM medium fermentation broth was diluted with sterile water into 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} fermentation broths in a concentration gradient. The mass concentration of DSF was continuously increased through this method, and the final enrichment of the strain was achieved. Then, 100 μL of diluted fermentation broth from each concentration gradient was evenly spread on LB solid plates and incubated at 30°C in an incubator, and single colonies were picked. The LB solid plate was repeatedly scribed and purified until a single strain was isolated (Bhatt et al., 2020; Zhang et al., 2022). The isolated single strains were stored in a refrigerator at −80°C.

Single colonies of the purified strains were inoculated in 40 mL of MSM basal medium with DSF as the sole carbon source, resulting in a final mass concentration of 2 mM DSF, then incubated at 30°C for 48 h in a 200 rpm shaker for DSF extraction and high-performance liquid chromatography (HPLC) determination of DSF residues. The strain with the highest DSF degradation rate was finally obtained, which was named F25.

Identification of *Burkholderia* sp. strain F25

The morphological characteristics of the colonies were studied. Strain F25 was scribed in LB solid medium and incubated at 30°C for 48 h. The genome of strain F25 was extracted as a template and 16S rDNA PCR amplification of the strain was performed using bacterial universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) (Li et al., 2022). The 16S rDNA gene sequence of strain F25, with a length of 1407 bp, was obtained using the above method and then compared with the NCBI (National Center for Biotechnology Information) database (http://www.NCBI.nlm.nih.gov/).

Antibiotic susceptibility analysis of strain F25

The antibiotic susceptibility of strain F25 was studied to better investigate the biocontrol potential of strain F25. A total of 50 μL of the bacterial solution was added to 5 mL of LB liquid medium, followed by the addition of antibiotics, where the final concentration gradient was 5, 10, 20, 50, 100, 150, 200, 250, 300, 350, and 400 $\mu g \cdot mL^{-1}$. Three sets of replicate trials were set up for each concentration gradient. Cultures were incubated at 30°C and 200 rpm for 16–24 h before measuring and recording the OD_{600} values of the cultures.

Determination of the relationship curve between growth and degradation DSF of strain F25

A single colony of strain F25 was inoculated in LB medium and pre-cultured to the logarithmic phase, following which the resulting broth was centrifuged at 4000 rpm for 5 min. The supernatant was discarded, and the organism was washed in 0.9% sterile saline, then re-suspended as seed suspension. The bacteria were then inoculated into 50 mL of MSM basal medium at an inoculum of 1:100 and DSF was added to a final concentration of 2 mM. The culture was incubated at 30°C for 72 h at 200 rpm, and samples were taken at regular intervals. The samples were collected at different time points, and the OD_{600} value was measured spectrophotometrically to indicate the growth of strain F25, while the residual amount of DSF was measured by HPLC to indicate the degradation of DSF by strain F25.

Antagonistic test of F25 and pathogenic bacteria

To study the antagonistic effect of strain F25 with pathogenic bacteria, studies were carried out on LB solid plates (Li et al., 2020; Zhou et al., 2022). A bacterial solution of *Xcc* was inoculated at 10% into the melted LB. After cooling, the LB agar plates containing the pathogen were punched with an inactivated punch, and 20 μL each of bacterial solution, metabolite solution, acetonitrile, and sterile water of strain F25 were injected into the punched wells. The plates were incubated at 30°C for 24 h. If antagonism occurred, hyaline circles would appear in the LB plate.

Study on the effect of strain F25 on the biological control of black rot of radishes

Single colonies of strain F25 and XC1, a DSF-dependent pathogen, were isolated and pre-cultured in LB medium until the logarithmic phase. The resulting broth was centrifuged at 4000 rpm for 5 min, and the supernatant was discarded. The organisms were washed with 0.9% sterile saline and re-suspended as seed suspension. The bacteria were then inoculated into LB medium at an inoculum of 1:100 and incubated at 30°C and 200 rpm until the logarithmic phase. The bacteria were re-suspended in PBS buffer to obtain suspensions of strain F25 and XC1.

The bacterial suspension of strain F25 was mixed with the suspension of XC1 to obtain the mixed bacterial solution. The fleshy roots of white radish were washed with distilled water, then sliced when the surface was dry. The fleshy roots were sliced

crosswise to obtain round slices about 0.3 cm thick and placed in Petri dishes (with cotton moistened with sterile water). The OD_{600} of both strain F25 and XC1 was 0.2. The mixture was coated with a spreading rod and incubated at 30°C for 48 h to observe the incidence. XC1 alone and F25 alone were used as positive and negative controls, respectively. In total, there were four experimental groups, divided into XC1+sterile water, XC1+F25 +sterile water, F25+sterile water, and sterile water. In the trays in which the radish slices were placed, we included moistened sterile wet water sponges and the trays were sealed with cling-film. The experimental procedure was repeated at least three times for each group. Incubation was carried out at 28°C for 48 h. The extent of the lesion was quantified by measuring the area of maceration, compared to the tissue without the lesion before inoculation (Liao et al., 2015; Liu et al., 2017).

Biocontrol experiments on the effect of *Burkholderia* sp. F25 crude enzyme on Xcc

The crude enzyme solution of strain F25 was prepared by the following method. The overnight culture of strain F25 was centrifuged at 4°C for 10 min at 10,000 rpm, and the resulting supernatant was the extracellular enzyme. Washing and suspending the cellular sediment was achieved by rinsing it three times with PBS. The cell suspension of strain F25 was then sonicated and centrifuged. The supernatant obtained was the intracellular enzyme to be collected. To verify its effect on the control of *Xcc* black rot, experiments were performed with the resulting crude enzyme extract, with four designs: (1) XC1-treated radish slices; (2) radish slices treated with 1 μl of XC1 and intracellular enzyme of strain F25; (3) treatment with 1 μl of extracellular enzyme of strain F25 and XC1 radish slices; and (4) radish slices treated with sterile water as a control. Three replicate trials were performed for all treatments. A final assessment of the disease level severity was performed.

Acylase activity test

To determine whether strain F25 presents acylase activity (Bao et al., 2020), we analyzed the degradative enzymes of F25 using an Acylase Activity Assay Kit (Solarbio, Beijing Solarbio Science & Technology Co., Ltd., China). Strain F25 was incubated in LB for 12 h. Intracellular and extracellular enzymes of strain F25 were extracted and collected separately. Four experimental groups were designed, with one intracellular enzyme and one extracellular enzyme setup, a negative control group without enzyme addition, a blank control group without enzyme addition, and a blank control group with distilled water. The acylase catalyzed the transfer of the acetyl group of acetyl coenzyme A to butanol and the simultaneous reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), to produce 2-nitro-5-thiobenzoic acid (TNB), which is a yellow compound.

Statistical analysis

Experimental data were analyzed by one-way analysis of variance (ANOVA), and means were compared by Bonferroni's multiple comparison test using the GraphPad Prism software (Version 6.0). Experiments were arranged as a completely randomized design, and $P < 0.05$ was considered to indicate statistical significance.

Results

Isolation, screening, and identification of strain F25

In this study, strains with DSF degradation ability were screened from the collected soil samples by enrichment culture. MSM with DSF as the sole carbon source was prepared and strains from different soil samples were enriched in the MSM. Nine morphologically different strains capable of degrading DSF were attained by streaking plate method and named as F20~28, respectively. Among them, strain F25 with rapid DSF degradation potential was selected and used in further studies. The F25 strain was deposited at the Guangdong Microbial Culture Collection Center (GDMCC) with the collection number GDMCC NO: 60346.

The above strain F25 was scribed in LB solid medium and incubated at 30°C for 48 h. As shown in Figure 1A, the colonies were yellowish-green in color, slightly elevated, and had smooth and opaque surfaces and neat edges. Strain F25 was diffusely turbid and aerobic in LB liquid medium. Scanning electron microscopy indicated that the strain was rod-shaped (or short spherical), with a size of 0.5–1.0 × 0.3–0.5 μm (Figure 1B).

The genome of strain F25 was extracted as a template, and 16S rDNA PCR amplification of the strain was performed using bacterial universal primers, in order to obtain the 16S rDNA gene sequence of strain F25, which was 1407 bp in length. It was compared with the NCBI database (http://www.NCBI.nlm.nih.gov/), and we found that strain F25 had 99% high homology with *Burkholderia cepacia* TC62 (AY677087.1). The standard strains in the BLAST results were compared, and a phylogenetic tree was constructed using MEGA 6.0. The constructed phylogenetic tree is shown in Figure 1C.

Therefore, strain F25 was identified as *Burkholderia* sp., in accordance with its morphological characteristics, 16S rDNA gene sequence, and phylogenetic analysis. The antibiotic susceptibility of strain F25 was further investigated. The results showed that strain F25 was resistant to 400 $\mu g \cdot mL^{-1}$ or more of gentamicin, neomycin, carbenicillin, ampicillin, and

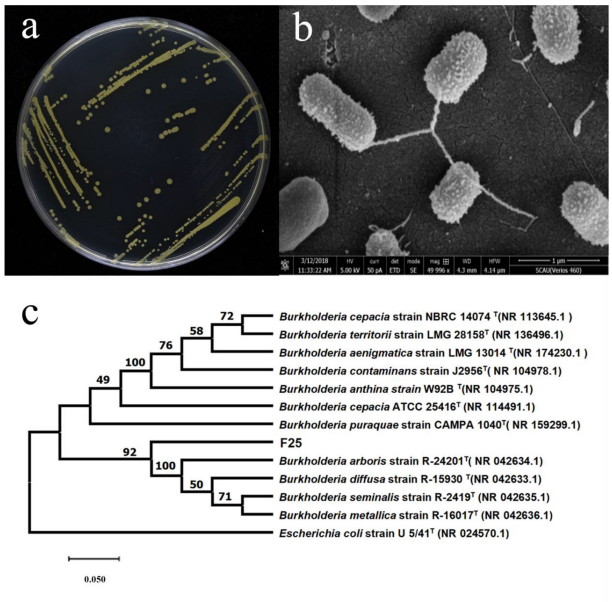

FIGURE 1
(A) Morphological characteristics of strain F25; **(B)** morphological characteristics observed under the scanning electron microscope (5000×); **(C)** Phylogenetic tree based on 16S rDNA sequences of *Burkholderia* sp. F25. Numbers in parentheses represent GenBank accession numbers. Numbers at nodes indicate bootstrap values. The bar represents sequence divergence.

streptomycin; 300 μg·mL^{-1} of kanamycin; and 50 μg·mL^{-1} of tetracycline and chloramphenicol (Figure 2).

DSF degradation kinetics

The DSF degradation ability of the strains was tested by growing the strains in MSM spiked with DSF. The solution was extracted at regular intervals and stored for the detection of residual DSF. The HPLC results are shown in Figure S1, from which it can be seen that the amount of DSF decreased with time and finally disappeared completely. At 12, 24, 36, 48, and 60 h, strain F25 degraded DSF up to 16.35%, 29.20%, 32.63%, 83.00%, and 100%, respectively. The growth and degradation curves of the corresponding strain F25 with DSF as the only carbon source are shown in Figure 3, where the degradation of DSF was

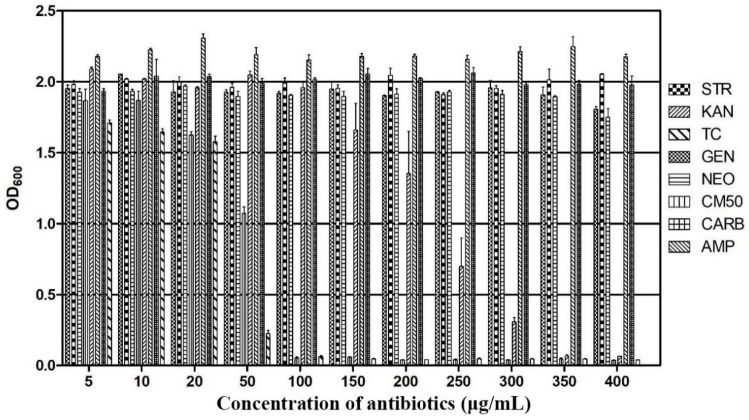

FIGURE 2
Antibiotic sensitivity of *Burkholderia* sp. F25. Strain F25 was resistant to 400 μg·mL^{-1} or more of gentamicin (GEN), neomycin (NEO), carbenicillin (CARB), ampicillin (AMP), and streptomycin (STR); 300 μg·mL^{-1} of kanamycin (KAN); and 50 μg·mL^{-1} of tetracycline (TC) and chloramphenicol (CM50).

positively correlated with the growth of the strain. In the presence of DSF, the strain growth had no lag period and rapidly entered the logarithmic phase of growth. The fastest phase of DSF degradation by this strain was 36-48 h and, when the strain was cultured to 60 h, the DSF was completely decomposed. The natural degradation rate of DSF in the control was 20% within 60 h.

Degradation products and pathways of strain F25

Strain F25 was inoculated into MSM medium spiked with DSF, and multiple time points were set for sampling, in order to investigate the pathway of DSF degradation by strain F25. Five metabolic products were identified during the biodegradation of DSF. Figure S2 shows the results of the GC-MS analysis. A distinct peak was detected at a retention time (RT) of 17.459 min with a characteristic mass fragment [M+] at m/z = 99.0 and a major fragment ion at m/z = 43.1; this important compound was identified as DSF (Figure 4A). With time, the peaks of DSF decreased and a new compound appeared, at a RT of 15.003 min with m/z = 73.0 as the base peak, which was characterized as n-decanoic acid, based on the elution time and how well the molecular ion matched the corresponding authentic compound in the NIST database (Figure 4B). In addition to this, we detected several degradation products of DSF (Figures 4C–F). It is worth noting that these metabolites were transiently present. Finally, DSF was completely decomposed into carbon dioxide and water.

The metabolic pathway of DSF in strain F25 was proposed based on the intermediates formed during degradation and the chemical structure of DSF, as follows. First, the degradation of DSF begins with the oxidation of branched carbon atoms to form fatty acids with one less carbon atom. Then, the *cis*-double bond of the formed intermediate is converted to a *trans*-double bond by isomerase. Through β-oxidation of the fatty acid, the intermediate product forms a *trans*-2-decenoic acid with two fewer carbon atoms. The unsaturated fatty acids then undergo hydrogenation to form saturated fatty acids. Finally, these compounds disappeared with the complete degradation of DSF by strain F25.

Antagonistic effect of strain F25 on XC1

Antagonism experiments were performed on strains F25 and XC1, in order to verify the presence of antagonism between them. LB agar plates containing pathogenic bacteria were prepared, and bacterial suspensions of strain F25 were injected into the plates. The results demonstrated that, when strain F25 was grown on the same plate as XC1, the plate showed no obvious transparent circle, indicating no zone of inhibition. The other two controls also showed no zone of inhibition. The antagonism experiment indicated that strain F25 has no antagonistic effect on *Xcc*.

Study on the effectiveness of strain F25 in the biological control of black rot

We tested the biocontrol ability of strain F25 against *Xcc* on radish slices, where the severity of the disease was reflected by the size of the maceration zone. The results are shown in Figure 5. The severity of the disease was high in radish slices treated with XC1 alone (Figure 5C). The dipping zone was significantly smaller in radish slices treated with the F25 and XC1 mixture (Figure 5D), while treatment of radish slices with strain F25 alone indicated that the strain was not harmful to the

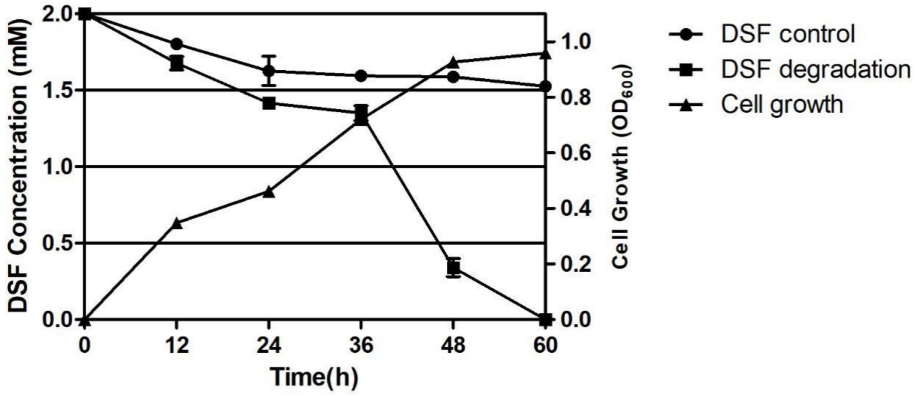

FIGURE 3
Degradation of diffusible signal factor (DSF) during the growth of *Burkholderia* sp. F25. Each experiment was conducted with three replicates. Bars indicate the standard deviation of the mean.

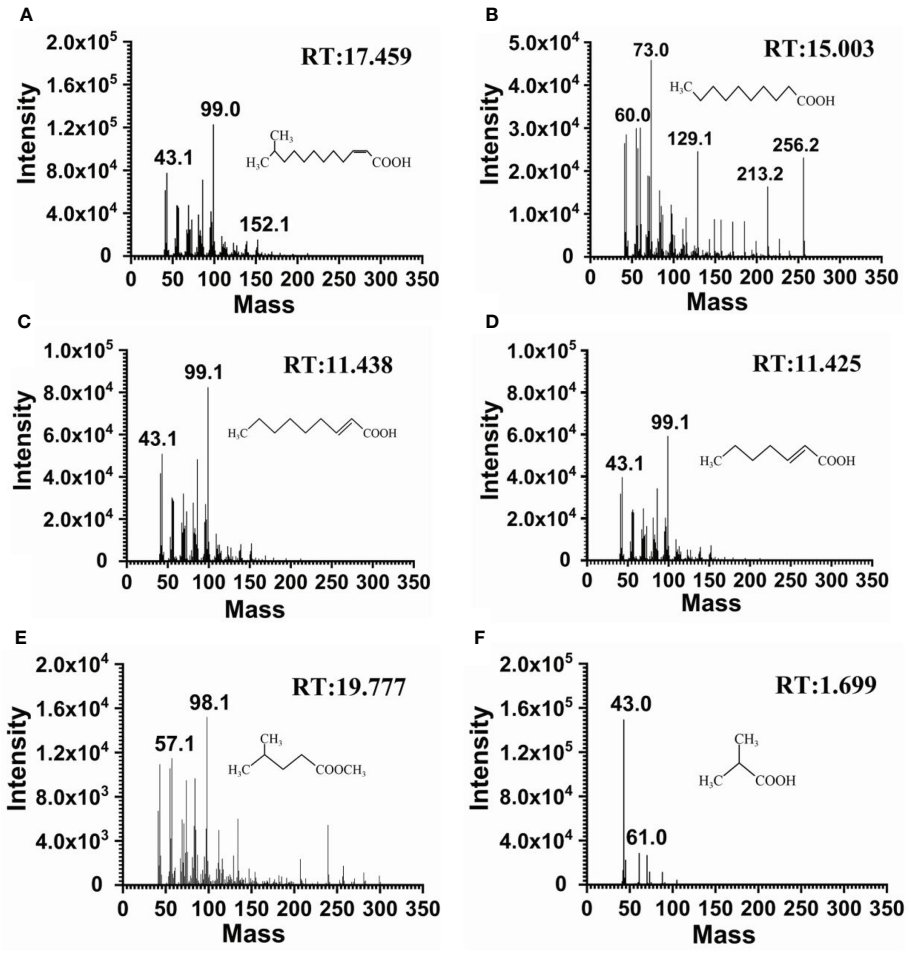

FIGURE 4
Mass spectrometric analysis of the DSF degradation products of *Burkholderia* sp. F25. GC-MS spectrum showed DSF (*m/z* of M+H = 99.0) with an HPLC retention time (RT) of 17.459 min **(A)**. GC-MS spectrum showed the major intermediate product *N*-decanoic acid (*m/z* of M+H = 73.0) with an HPLC retention time (RT) of 15.003 min **(B)**. GC-MS identified the mass spectra and structures of the other degradation products detected: *trans*-2-nonenoic acid **(C)**; *trans*-2-heptenoic acid **(D)**; methyl 4-methylpentanoate **(E)**; and 2-methylpropanoic acid **(F)**.

radishes (Figure 5B). Treatment of radish slices with only distilled water did not show any disease (Figure 5A). These results indicate that strain F25 exhibited good biological control potential against *Xcc* and, so, can be used as a biological agent to prevent infection by bacterial plant diseases.

Biocontrol efficiency of F25 crude enzyme

To verify the biocontrol effect of strain F25 crude enzyme on *Xcc*, the pathogenic bacteria XC1, intracellular enzyme, and extracellular enzyme were inoculated onto radish slices under ex vivo conditions and incubated for 48 h. The experimental results are shown in Figure 6, where a slight decay was observed in the crude enzyme-treated group; however, the area of the macerated zone was significantly lower, compared to the XC1-treated group. These results indicate that the crude enzyme of strain F25 also has a good biocontrol effect and has great potential to be developed for the treatment of plant diseases caused by *Xcc*.

F25 has acylase activity

To verify whether strain F25 presents acylase activity, intracellular and extracellular enzymes of strain F25 were extracted and tested using an analytical kit. From the results, we observed that the control group was colorless, while the experimental group with the addition of the intracellular enzyme exhibited a distinct yellow color (Figure S3), indicating that strain F25 possesses acylase activity.

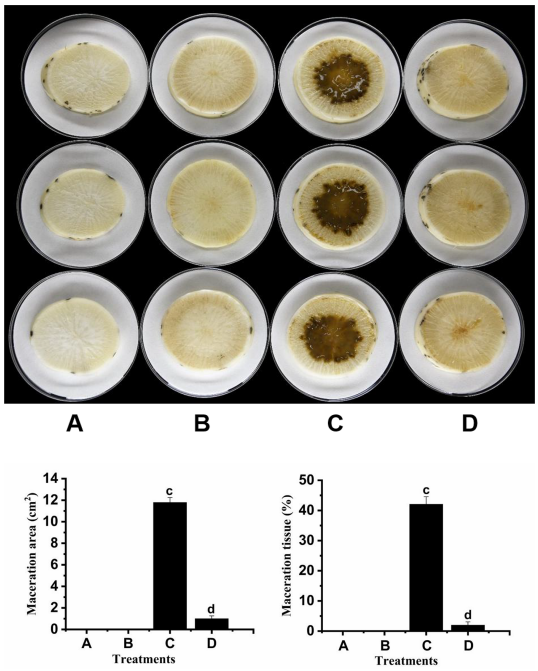

FIGURE 5
Biocontrol test of *Burkholderia* sp. F25 against black rot disease on radish slices under laboratory conditions. **(A)** shows radish root slices inoculated only with distilled water; **(B)** shows radish root slices inoculated only with the suspensions of DSF-degrading strain F25; **(C)** shows radish root slices inoculated only with the suspensions of black rot pathogen *Xcc*; **(D)** shows radish root slices co-inoculated with the suspensions of *Xcc* mixed with F25. Experimental data were analyzed by one-way analysis of variance (ANOVA), and means were compared by Bonferroni's multiple comparison test using the GraphPad Prism software (Version 6.0). Experiments were arranged as a completely randomized design, and $P < 0.05$ was considered to indicate statistical significance. Values with different small letters are significantly different according to Duncan test at $P < 0.05$ level.

Discussion

QS acts as a communication mechanism for microorganisms, regulating cell growth, secretion of pathogen virulence factors, and biofilm formation (Miller and Bassler, 2001; Deng et al., 2013a; Xu et al., 2021). All of these regulatory processes help microorganisms to cause disease and, thus, have a significant impact on agricultural production, the environment, and human health (Deng et al., 2013b; Whiteley et al., 2018). The QQ pathway can be used to inhibit the expression of pathogenic genes in the QS process by degrading the signaling molecules produced during the QS process by QQ bacteria or QQ enzymes (Fan et al., 2020), such that the signaling molecules do not reach a certain threshold value, thus blocking intercellular communication between the pathogenic bacteria (Boon et al., 2008). To date, studies on QQ bacteria have mainly focused on AHL-mediated QS family systems, and only a few studies have reported on the role of DSF-degrading bacteria in the control of black rot caused by *Xcc* in wild rape (Huedo et al., 2018; Samal and Chatterjee, 2019). A large number of microorganisms that can degrade signaling molecules exist in nature, which has the advantages of being diverse, abundant, and easy to cultivate (Zhang et al., 2021). Therefore, screening QQ bacteria from nature for biodegradation applications has obvious advantages.

In this study, we isolated a new QQ strain from an agricultural field, where morphological characteristics and 16S rDNA phylogenetic analysis of the strain identified it as a member of *Burkholderia*. Strain F25 was isolated from the soil of a perennial cultivated sweet potato field, and was well-adapted to the environment. QQ strain F25 can degrade DSF rapidly and efficiently and possesses acylase activity, as well as having a metabolic pathway by which DSF can be completely degraded and metabolized. Our experimental results showed that strain F25 can degrade DSF in a short period of time and can significantly reduce the function of DSF-dependent diseases to achieve a biological control effect. Previous studies have demonstrated that *Burkholderia* strains have strong metabolic capability and environmental versatility as well as excellent ability to manage

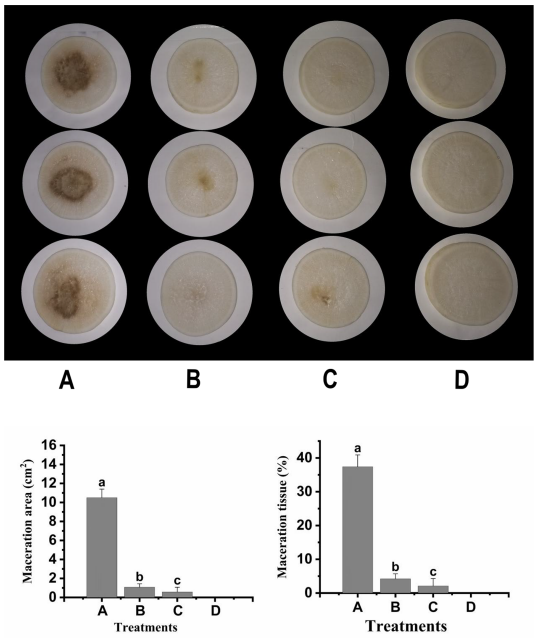

FIGURE 6
Preliminary biocontrol test of crude enzymes of *Burkholderia* sp. F25 against *Xanthomonas campestris* pv. *campestris* XC1: **(A)** Panel A, XC1 alone on plant slices; Panel **B**, XC1 + Intracellular enzyme; Panel **C**, XC1 + Extracellular enzyme; Panel **D**, Sterile water. **(B)** Maceration area (1) and maceration tissue (2) in each treatment. Experimental data were analyzed by one-way analysis of variance (ANOVA), and means were compared by Bonferroni's multiple comparison test in the GraphPad Prism software (Version 6.0). Experiments were arranged as a completely randomized design, and $P < 0.05$ was considered to indicate statistically significant. Values with different small letters are significantly different according to Duncan test at $P < 0.05$ level.

bacterial and fungal pathogens infecting crop plants (Mannaa et al., 2018; Ye et al., 2020a; Pal et al., 2022). Furthermore, a new degradation pathway of DSF in the presence of microorganisms was proposed, based on the metabolites of isolate F25 detected by GC-MS and the chemical structure of DSF. First, to promote β-oxidation, DSF is converted from *cis* to *trans* double bonds, as catalyzed by enzymes, where the methyl groups on fatty acids are oxidized. Immediately afterward, β-oxidation continues to form intermediate products with two fewer carbon atoms. The unsaturated fatty acids are then hydrogenated to form saturated fatty acids. DSF is eventually degraded to carbon dioxide and water by microbial metabolism, without any long-term accumulation of intermediates. In addition, strain F25 presented significant resistance to gentamicin, neomycin sulfate, carbenicillin, ampicillin, and streptomycin.

Inoculation experiments were conducted to evaluate the biocontrol effect of strain F25. The results showed that strain F25 did not cause the development of other diseases and also had a good biocontrol effect against black rot caused by *Xcc* in plants. The above experiments demonstrated that strain F25 and its crude enzyme can be applied as effective biocontrol agents for controlling plant diseases caused by DSF-dependent bacterial pathogens, and the great potential of strain F25 for the control of DSF-mediated pathogenic bacterial damage was demonstrated. This provides a new avenue for the development of a treatment strategy that replaces chemical control with biological control, allowing for the blockage of QS without causing selection pressure.

Conclusions

In summary, we identified a novel quorum quencher, *Burkholderia* sp. F25, which can be used as a new QQ strain and has excellent DSF degradation capacity. Strain F25 possesses acylase activity and a pathway for complete DSF degradation and metabolism. In addition, strain F25 and its crude enzyme were able to significantly attenuate black rot in plants, and thus could be used as potential biocontrol agents against plant diseases caused by DSF-dependent bacterial pathogens. This work provided a biochemical basis for the efficient DSF-degrading activity of strain F25, and offers new perspectives for further studies on the suppression mechanisms of plant pathogens. In the future, in-depth studies on the gene clusters of strain F25 related to DSF degradation are required, in order to elucidate the genetic mechanisms of strain F25 and to improve its stability in practical applications.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding authors.

Author contributions

L-HZ, SC, HW, and LL conceived the presented idea. HY, W-JC, and JH contributed to the writing and prepared the figures. KB, ZZ, XZ, SL, SC, HW, and LL participated in revising the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor PB declared a past co-authorship with the authors. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1071693/full#supplementary-material

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EDITED BY
Muhammad Musa Khan,
Zhejiang University, China

REVIEWED BY
Kalpana Bhatt,
Purdue University, United States
Babar Iqbal,
Jiangsu University, China
Muhammad Hafeez,
Zhejiang University, China

*CORRESPONDENCE
Yuxing An
anyuxing@hotmail.com

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Physiological responses and antibiotic-degradation capacity of duckweed (*Lemna aequinoctialis*) exposed to streptomycin

Weijuan Huang, Rui Kong, Lijun Chen and Yuxing An*

Institute of Nanfan and Seed Industry, Guangdong Academy of Sciences, Guangzhou, China

Aquatic plants are constantly exposed to various water environmental pollutants. Few data on how antibiotics affect duckweed health and its removal ability. The aim of this study was to investigate the impact of streptomycin on the physiological change and uptake capability in duckweed (*Lemna aequinoctialis*) after exposure at different time points (0, 5, 10, 15 and 20 days). Duckweeds were exposed to streptomycin at a range of concentrations (0.1-10 mM). Results indicated that the high streptomycin concentrations (≥1 mM) resulted in a lower duckweed biomass (21.5-41.5%), RGR (0.258-0.336 g d^{-1}), decrease in total Chl and increase in carotenoids. Antioxidative enzymes, including CAT (18-42.88 U mg $protein^{-1}$), APX (0.41-0.76 U mg $protein^{-1}$), and SOD (0.52-0.71 U mg $protein^{-1}$) were found to accumulate in the streptomycin groups in comparison to the control group. The significant reduction (72-82%) in streptomycin content at 20 d compared to the control (40-55%) suggested that duckweed has a high ability in removing streptomycin. Transcriptome analysis showed that the secondary metabolic pathways including phenylpropanoid biosynthesis and flavonoid biosynthesis were significantly upregulated in the streptomycin setup compared to the control. Therefore, our findings suggested that duckweed can contribute to the streptomycin degradation, which should be highly recommended to the treatment of aquaculture wastewater and domestic sewage.

KEYWORDS

antibiotics, duckweed, plant physiology, water contamination, aquatic environment

Introduction

In recent years, the wide application of antibiotics has caused great risks to water quality, food and ecological safety after being released into the environment (Kovalakova et al., 2020). Various antibiotics have been found in groundwater, and domestic water that has become a challenge in aquatic ecosystems and could pose serious risks to human health (Jafari Ozumchelouei et al., 2020; Zeng et al., 2022). Although China's agricultural sector used less than 30,000 tons of antibiotics between 2014 and 2018 (Schoenmakers, 2020), the overall effects on the aquatic ecosystem requires more research. With attentions on environmental protection, there are more and more reports on the effect of antibiotics on the environment and aquatic plant. Sacher et al. (2001) investigated the status and hazards of antibiotic pollution in the environment, analyzed 108 groundwater samples in Germany, and found that 60 kinds of antibiotics in the water samples were detected. Zhang et al. (2017) has also detected quinolones, as well as sulfa and tetracycline in the soil of the Pearl River Delta in China. The impacts of quinotone antibiotics on aquatic environment was investigated, and results showed that quinotone were widely present in the sediments from Yang River and its estuary, and were observed in all five wetland plants (Liu et al., 2020). Tang et al. (2022) studied the evaluation of antibiotics bioaccumulation and trophodynamics in aquatic food webs, and found that metabolic biotransformation plays a significant role in driving biomagnification of antibiotics. Li et al. (2020) reported that a total of 83 target antibiotics were quantified in water and sediment samples collected from the Qinghai Lake, the largest inland lake of China, which suggests the urgent need to investigate the possible long-term enrichment and environmental risks of antibiotics in inland lakes. The findings of Huang et al. (2022) for the first time revealed that the antibiotic synthetic potential in activated sludges could aggravate environmental pollution.

Streptomycin is an aminoglycoside antibiotic with a molecular formula $C_{21}H_{39}N_7O_{12}$. Due to its simple availability and cost effectiveness, streptomycin is usually used alone or in combination with other antibiotics to treat bacterial diseases (Lyu et al., 2019). Streptomycin is also widely used in agricultural production to prevent and control plant diseases caused by various pathogenic microorganisms (Walsh et al., 2014). However, its abuse can cause residual drugs to enter the ecosystem and the human body through bio-enrichment and food chain methods, induce various side effects, cause gene mutations, and even cause cancer, which seriously threatens the ecological balance and human health (Yilmaz and Özcengiz, 2017; Wang et al., 2022). Risk assessment of streptomycin residues towards aquatic organisms of various biological processes remains unclear.

Duckweed has 37 species in the world, belonging to five genera including *Lemna, Spirodela, Wolffia, Wolffiella*, and *Landoltia* (Wang et al., 2021). *Lemna aequinoctialis* is a common and widely distributed aquatic plant in China, which has been extensively used in the research of plant biology, aquatic ecotoxicology, and water pollution remediation due to its rapid reproduction, high yield and rich nutritional value (Kummerová et al., 2016; Ekperusi et al., 2019). *Spirodela polyrhiza* has been reported to be able to remove a few antibiotics efficiently (Singh et al., 2018; Singh et al., 2019). After seven days, the medium containing duckweed had significantly lower residual ofloxacin content than the control (Singh et al., 2019). *Lemna paucicostata* removed more than 97% of hydrocarbons from wetlands after 120 days (Ekperusi et al., 2020). Halaimi et al. (2014) found that cadmium and methyl parathion could be removed with both *Lemna gibba* and *Lemna gibba* powder. Baciak et al. (2016) reported that *L. minor* takes up tetracycline in water reservoirs meanwhile the antibiotic significantly affects the duckweed's metabolism. Most target substances were adequately removed by a continuous-flow *Lemna minor* system with removal rates ranging from 26% (4-methyl-1H-benzotriazole) to 72% (5-chlorobenzotriazole) (Gatidou et al., 2017).

As antibiotic water pollution is gaining prominence as a global issue, the demand for evaluation on its toxicity, mechanism, and remediation has attracted increasing attention. Study on the degradation of streptomycin in water was found consistent with the first-order model in aquatic environments (Shen et al., 2017). Effects of streptomycin on growth of algae indicated that streptomycin is toxic to fresh algae, affects photosynthesis-related gene transcription (Qian et al., 2012). In terms of determining whether streptomycin is harmful to *L. aequinoctialis*, there is currently a lack of information in the scientific literature. Therefore, we carried out a toxicology experiment on duckweed using different concentrations of streptomycin and detected its changes from the physiological and molecular level. The aim of our study was to examine the effects of streptomycin on the duckweed system in terms of antibiotic removal efficiency, growth response, activity of antioxidant enzymes, or relevant physiological and degradation mechanism. Through the evaluation of this study, the potential streptomycin's toxicological effects on *Lemna aequinoctialis* are expected to be determined.

Materials and methods

Sample collection and preparation

The duckweed, *Lemna aequinoctialis* was harvested from a local pond near to the institute and further identified using two DNA barcodes *atpF-atpH* and *psbK-psbI* (Borisjuk et al., 2015). The sterilized duckweeds were cultured on half-strength Schenk & Hildebrandt (SH) basal salt mixture, pH 6, supplemented with

5 g/L glucose. The sterilized duckweed was performed using the method adopted from Huang et al. (2020).

Evaluation of duckweed toxicity

Different concentrations (0.1, 0.5, 1, 5, and 10 mg $L^{-1,}$ labeled as L5, L4, L3, L2, and L1) of streptomycin in the medium was set as per the methodology of Singh et al. (2019). The 1 g L^{-1} stock solution of streptomycin was made with methanol and 0.1x PBS. The stock solutions were diluted with 0.5x SH medium to creat various streptomycin concentration groups. A half-strength SH medium setup without streptomycin was used as a control. For each concentration, a separate control system (0.5x SH medium + antibiotics without duckweed) was set up to detect changes in photodegradation and hydrolysis other than phytodegradation in antibiotic degradation, which the treatment method was adopted from that described in Singh et al. (2018 and 2019). In a baby jar, we prepared 100 mL of sterile 0.5x SH media (CultureJar™ G9, cat.# C1770; PhytoTechnology Labs, KS, USA) covered with a thin layer of duckweed, and for each concentration of antibiotic with three replicates. The duckweed cultivation was using the method adopted from Huang et al. (2020). The growth period lasted for 30 days, and duckweeds treated with each concentration of antibiotic were harvested respectively at a set time points (0, 5, 10, 15 and 20 days). To maintain a constant growth volume, distilled water was used to replace any evaporation-related water loss during the experiment.

Duckweed biomass and biochemical analysis

Fresh duckweeds were harvested from the experimental groups every five days, and the change in plant biomass in each group was measured. The RGR (relative growth rate) in all setups at each timeline was measured using the equation RGR = $(Dbt_d - Dbt_0)/NA$, as described by Verma and Suthar (2014). Where, Dbt_d and Dbt0 are the fresh biomass of duckweed recorded every 5 days (5 d, 10d, 15d, and 20d) as well as 0 d, respectively; N is the number of days in the experiment, and A is the baby jar's medium's surface area.

The method was used to measure the total chlorophyll and carotenoid content of leaves (Lichtenthaler, 1987). The protein content of harvested duckweed (0.1g) was quantified with a total protein quantitative assay kit. The CAT, SOD, and APX enzyme assay kits were used to quantify catalase, superoxide dismutase, and ascorbate peroxidase, respectively.Analysis of residual streptomycin

Each group's remaining medium was filtered through a membrane filter of 0.45 m before being collected on days 5, 10, 15, and 20. Streptomycin was detected using HPLC-MS/MS, which was performed using the method adopted from Wang et al. (2019).

RNA sequencing and analysis

Duckweeds with and without streptomycin treatment were collected on day 5 for RNA sequencing. Total RNA changes after antibiotic treatment were examined using transcriptomic analysis. In order to perform high-throughput sequencing, total RNA was extracted, and the sequences were processed and analyzed followed by Novogene company (Beijing, China). According to the methods of Huang W, et al. (2021), one microgram of total RNA with RIN values above 6.5 was used for next-generation sequencing library preparation. Then, libraries with different indices were multiplexed and loaded on an Illumina HiSeq instrument according to manufacturer's instructions (Illumina, CA, USA).

Data analysis

Each treatment's solution and duckweed samples were used to calculate the mean values and standard deviation (SD) for all three replicates. The exposure levels of antibiotics in solution were calculated using time-weighted average concentrations to express the actual exposure concentrations. All data were compared using SPSS 26.0 (SPSS, Chicago, IL, USA) using repeated measure ANOVA (RMANOVA). The LSD test was used to find significant differences between the analysis variables ($p < 0.05$).

Results

Antibiotic-induced changes in biomass and photosynthetic pigments

As the concentration of streptomycin rises, duckweed's biomass and photosynthetic pigments tend to decrease in setups compared to the control. The biomass between experimental group and control group at each batch of the experiment was significantly different ($P<0.05$). The harvested duckweed biomass in different treatment setups was found to be ranged between 0.117 g (1 mg/L) and 0.72 g (0.01 mg/L) on day 20 (Figure 1A). The experimental group had a greater reduction in biomass exposed to a higher concentration of streptomycin (≥ 5 mg L^{-1}) than the control group. At a higher tested concentration (≥ 5 mg L^{-1}), L1 and L2 showed a modest but statistically significant inhibition of their RGR from day 10, and they had 41.5% and 21.5% less biomass (day 20) compared to the initial weight (0.2g), respectively. While, the harvested duckweed biomass became increased significantly on day 15 by 63.5%, 76.5%, and 145% in L3, L4 and L5, respectively. The final biomass on day 20 was in the following order: CK > L5 > L4 > L3 > L2 > L1 (Figure 1A).

The values of the relative growth rate (RGR) in all streptomycin treatment groups were significantly lower than

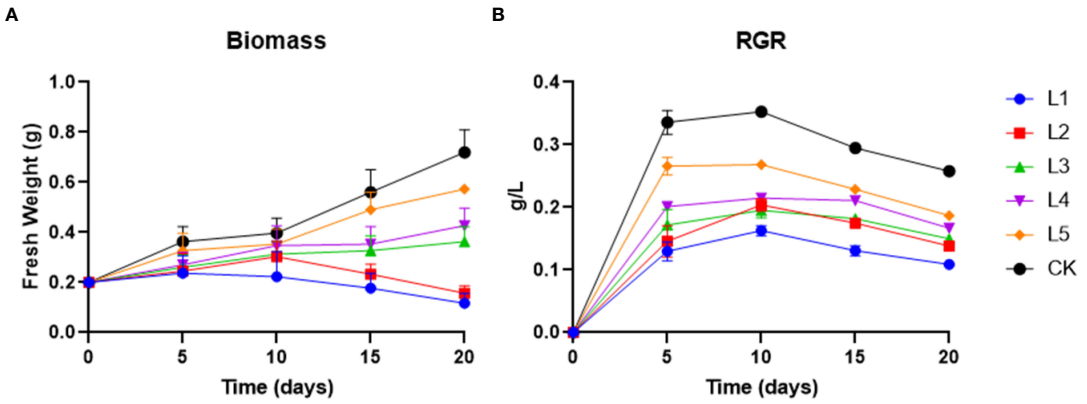

FIGURE 1
(A) Biomass and **(B)** RGR were recorded over four time courses in various streptomycin and control setups. L1, L2, L3, L4, and L5 represented for 10, 5, 1, 0.5, and 0.1 mg L^{-1} concentration of streptomycin, respectively (mean ± SD, n= 3).

those of the control (0.258~0.336 g d^{-1}) group at the end (Figure 1B). For the treatment setups within 10 days, the RGR showed an increment whereas, the RGR was in negative scale in the following ten days. RGR values in each experimental time course showed statistically significant differences between multiple groups. The RGR recorded in L1, L2, L3, L4 and L5 was found to be 0.13, 0.146, 0.172, 0.201 and 0.266 g d^{-1}, respectively, compared to the control after 5 days of cultivation (Figure 1B).

On day 5, the high dose of streptomycin (10 mg L^{-1}) resulted in a significant decrease in total Chl (28.2%), but the subsequent days of cultivation did not show any significant changes (Figure 2). Similar to the other four experimental setups, the total Chl content in L1 was trending downward, and the content of total Chl in the end of experiment was in the following order: CK > L4 > L5 > L2 > L1 > L3 (Figure 2A). From 0.06 mg g^{-1} in the initial group to 0.13 mg g^{-1} in the group exposed to the highest streptomycin concentration, the carotenoids content significantly increased (Figure 2B). The ratio of carotenoids/total Chl increased considerably in setups of streptomycin, suggesting that streptomycin continuously changed the content of photosynthetic pigments.

Effects of streptomycin on anti-oxidative enzymes in duckweed

All duckweed setups of various streptomycin concentrations were subjected to a four-time course measurement of the anti-oxidative enzyme activities to determine how streptomycin

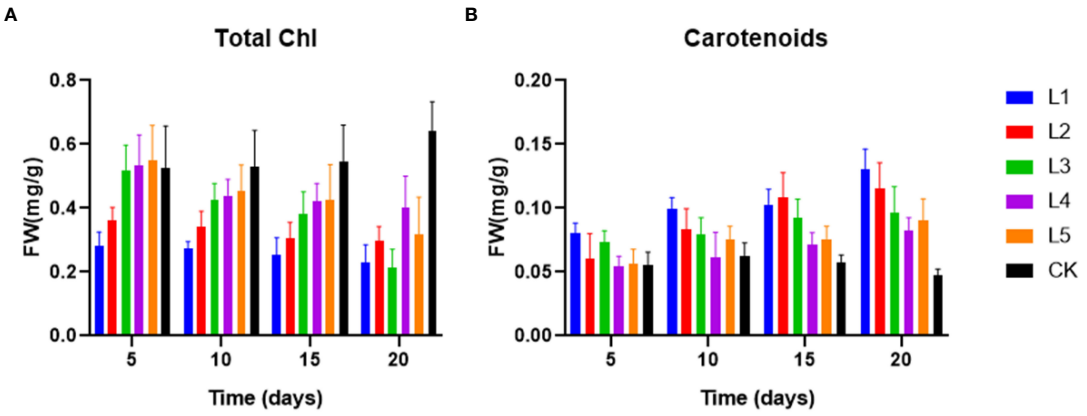

FIGURE 2
The content of photosynthetic pigments: total Chl **(A)** and carotenoids **(B)** in fronds of *Lemna aequinoctialis* exposed to different concentration of streptomycin in medium.

affected duckweed. The results showed that the streptomycin-treated duckweeds showed an increased activity of SOD, CAT, and APX compared to the control (Figure 3). After 10 days of cultivation, the differences of three enzyme activities between different experimental groups became gradually increased. SOD activity increased from 0.52 U mg protein^{-1} to 0.71 U mg protein^{-1} in duckweed setups containing streptomycin by the end of the experiment, indicating a plant anti-oxidative response (Figure 3A). Initially, duckweed was significantly inhibited in activity when exposed to high concentrations of streptomycin (≥1 mg L^{-1}). As the exposure time increased, the SOD activity in most experimental groups became increased to varying degrees. After exposure for more than two weeks, the SOD activity in duckweed was significantly increased only in a few groups (L1 and L2).

The CAT activity profile showed a different trend. The CAT activity of duckweed tissues did not change with the treatment of 0.1 mg L^{-1} streptomycin. Catalase activity increased gradually during the initial 5 days and afterward grew rapidly from day 5 to day 20 with the exception of the CK and L5 group. CAT activity was the highest (42.88 U mg protein^{-1}) in response to 10 mg L^{-1} of the streptomycin on day 20. The CAT showed a significant difference between the various treatments, and multiple comparison tests displayed a significant difference between day 10 to day 20 (Figure 3B). Streptomycin can affect the CAT activity of duckweed, and the influence level would be relevant with the concentration of streptomycin and the time of exposure to streptomycin. Especially, when the concentration of streptomycin reached to 1mg/L, the CAT activity of duckweed was significantly inhibited. Diverse streptomycin concentrations significantly affected duckweed' APX activity, and became increased with higher concentrations of streptomycin (Figure 3C). The APX activity continued to increase with the increase of streptomycin dose (L5 < L4 < L3 < L2 < L1, 0.41-0.76 U mg protein^{-1}).

Removal of streptomycin in duckweed reactor

In order to determine how the antibiotic would behave in the medium when duckweed was present, all batch experiments detected the residual concentration of streptomycin. The results showed that the amount of streptomycin in the duckweed setups

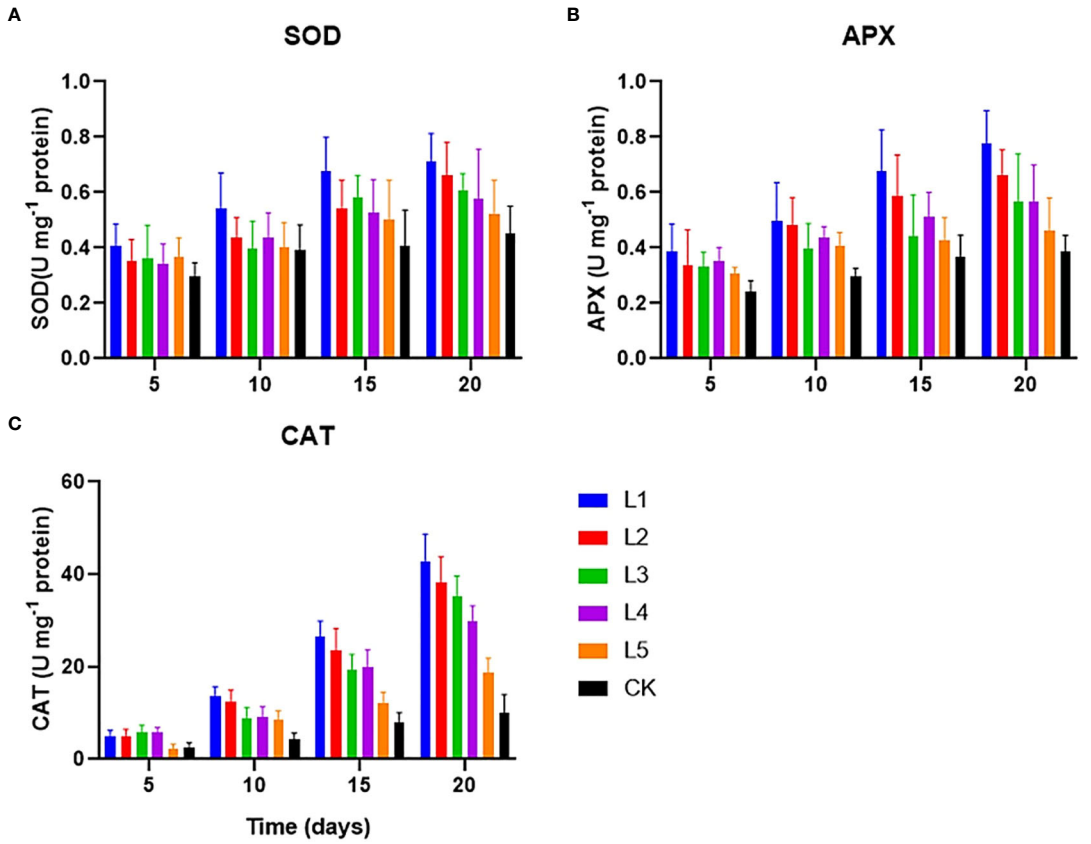

FIGURE 3
The anti-oxidative enzymes [**(A)** SOD, **(B)**. APX and **(C)** CAT] in *L. aequinoctialis* exposed to a variety of streptomycin concentrations in the medium.

decreased by 72% to 82% and in the control by 40% to 55% at the end of the experiment (20 days) (Figure 4). In the duckweed setup, the remained concentration of streptomycin at the end of the experiment was L1 > L2 > L3 > L4, indicating that the complete reduction of the antibiotic in the media at a high concentration required more time than that of the low concentration of the antibiotic. The absence of a streptomycin concentration in the L5 group (0.01 mg) indicated that the streptomycin in the solution has been completely degraded. Another possibility could be caused by the detection limit of the instrument used.

Transcriptomics profiling of duckweed in response to streptomycin

A total of 21,368,136 and 20,568,117 raw reads were obtained from the experimental group (YN) and control group (CK), respectively. Clean reads 20,363,940 and 19,646,041 were retained and used for assembly after low-quality sequences were removed. The RNA-Seq results showed that YN and CK had 329 differentially expressed genes (DEGs). When compared to the CK group, the YN group had a total of 144 genes that were significantly upregulated and 185 genes that were significantly downregulated (Figure 5A and Supplementary Data). The similar expression patterns observed in nine DEGs (Supplementary Table 1) selected at random for qRT-PCR verification confirmed the validity of the transcriptome sequencing data.

Utilizing the GO (Gene Oncology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) databases, we carried out two pathway-based analyses to demonstrate the connection between DEGs and metabolic pathways. An overview of the metabolic changes that occurred in the biological processes of duckweed in response to antibiotics in the aquatic environment was provided by the KEGG enrichment analysis. KEGG enrichment analyses showed that phenylpropanoid biosynthesis, flavonoid biosynthesis, photosynthesis, stillbenoid, and diarylheptanoid were significantly enriched in YN compared to CK (Figure 5B). In the YN group, the key genes in the pathway of phenylpropanoid biosynthesis including CYP84A, CYP73A, and CYP98A were significantly downregulated compared to those in the CK group. Similarly, genes in the pathway of flavonoid biosynthesis including CYP73A and CYP98A were significantly downregulated in YN compared to CK. The key genes LHCB1 and LHCB6 in the pathway of photosynthesis were significantly downregulated in YN compared to CK. Functional annotations of DEGs based on the GO pathway analysis showed a number of important metabolic pathways involving oxidoreductase activity, stress response, and growth that were significantly altered in YN compared to CK (Figure 5C).

Discussion

The issues of food safety and environmental pollution brought by the excessive use of antibiotics are becoming increasingly alarming, despite the fact that antibiotics play a crucial role in protecting human health and encouraging the growth of animal husbandry (Ezzariai et al., 2018; Anjali and Shanthakumar, 2019; Zeng et al., 2022). Phytoremediation is an emerging biological technology that aids in the removal of harmful pollutants from water bodies and soil; however, research on phytoremediation requires a comprehensive understanding of processes and mechanisms (Huang D, et al., 2021). Duckweed, as a globally widespread aquatic plant, is attractive for phytoremediation of pollutants in water bodies (Hu et al., 2021; Walsh et al., 2021; Cai et al., 2022). Studies have

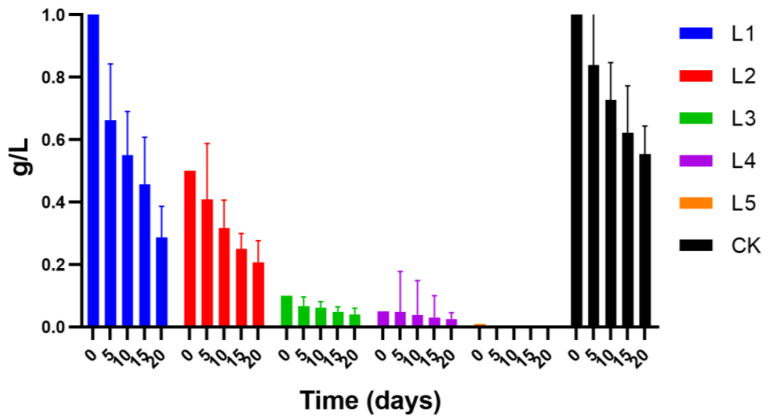

FIGURE 4
Various concentrations of streptomycin removal by duckweed system.

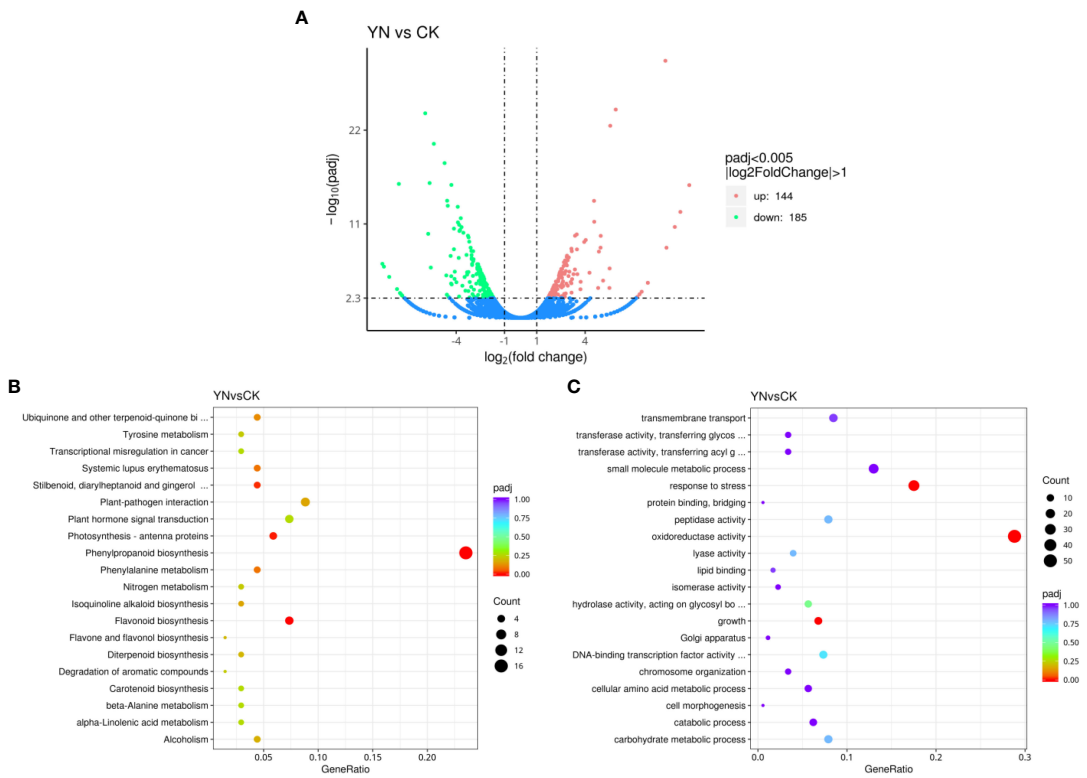

FIGURE 5
Numbers of differentially expressed genes (DEGs) in duckweed between YN (in response to streptomycin) and CK **(A)**. KEGG **(B)** and GO **(C)** pathway enrichment for DEGs in YN *vs.* CK.

revealed that duckweed has a great potential in the bioremediation of eutrophic water bodies, heavy metals, antibiotics, insecticides, and organic pollutants (Ceschin et al., 2020; Yang et al., 2020a; Farid et al., 2022; Maldonado et al., 2022). However, whether duckweeds can accelerate the degradation of streptomycin and what are their underlying physiological mechanisms are rarely reported. Based on physiological and molecular biological methods, this study revealed that streptomycin had a severe toxicological effect on the physiological metabolism of duckweed, and found the phytoremediation potential of duckweed on the removal of streptomycin from water body.

The continuous 20-day antibiotic toxicity assessment revealed that streptomycin was toxic to common duckweed at the lowest concentration (0.1 mg L^{-1}), resulting in a nearly 15% decrease in growth rate and biomass yield (Figure 1). The yield and growth rate of duckweed were both reduced by 45% when streptomycin was applied at a concentration of 10 mg L^{-1}. In all of the setups, *Lemna aequinoctialis* did not cause chlorosis to affect the fronds. However, by the end of the experiment, the setups with a higher concentration of streptomycin clearly showed that the fronds had died, suggesting that the antibiotic had a toxic effect on duckweed at a high concentration. This could be explained by the extremely high percentage (93%) of dead mitochondria in duckweed caused by antibiotics in the findings of (Krupka et al., 2021). Maldonado et al. (2022) further revealed that the metabolization of antibiotics occurs in three phases in duckweed which contribute to the removal of antibiotics residues in water. However, the process of antibiotic degradation in duckweed is accompanied by the increase of antioxidant enzymes, slower growth, reduced photosynthesis and other physiological processes (Yang et al., 2020a; Farid et al., 2022), which can be regarded as a cost of adapting to the environmental pollution.

As is reported, streptomycin is an inhibitor of chlorophyll synthesis (Mancinelli et al., 1975). Some abiological factors can stimulate the increase of carotene content, and the function of carotene is to enhance plant resistance under stress conditions. By reducing active reaction centers and preventing electron transport in photosynthetic system II, streptomycin may hinder plant growth (Zhang et al., 2019). In the high dose of streptomycin, the results showed that the total amount of chlorophylls decreased and the amount of carotenoids significantly increased (L1 and L2) (Figure 2). In our study, different concentrations of streptomycin can lead to a significant increase in carotenoid content in duckweed, suggesting that

duckweed is less resistant to streptomycin stress. Similar results were also found in other aquatic plants, including *Chlorella vulgaris* (Perales-Vela et al., 2016) and *Microcystis aeruginosa* (Qian et al., 2012). Additionally, it has been observed that streptomycin inhibits the synthesis of chlorophyll and carotenoids in the germination of barley seeds (Yaronskaya et al., 2007).

Streptomycin has high water solubility, is easily absorbed by plants, affects the normal physiological metabolism of plants, inhibits plant DNA replication, and thus produces genotoxic effects (Qian et al., 2012). Based on the environmental sensitivity model material duckweed, our study has shown that when given a high dose (0.1 to 10 mg L^{-1}) that is appropriate for the environment, streptomycin has a significant negative impact on duckweed's cellular metabolism and gene expression. According to the transcriptomics data, 329 DEGs were primarily found on the KEGG pathways for phenylpropanoid and flavonoid biosynthesis, which significantly enriched in YN *vs*. CK (Figure 5), suggesting that the enriched secondary metabolic pathways may improve duckweed stress resistance. However, there are a lot of relevant genes in plant species that are responsible for the biosynthesis of flavonoids and phenylpropanoid, thus more research is needed to find the target genes and verify their expression. In addition, Hu et al. (2021) clarified the metabolism profiles of the phyllosphere and rhizosphere microbes, providing a fresh perspective on the effects of antibiotics on livestock wastewater through the duckweed system. Therefore, mining the mechanism of plant degradation of pollutants at the molecular level still requires in-depth research.

To the best of our knowledge, there is no published study on how streptomycin affects the duckweed. Regarding the effect of antibiotics on aquatic plants, it is confirmed that antibiotics have a certain threat to aquatic plants, but different aquatic plants have different responses to various types of antibiotics (Harrower et al., 2021; Xu et al., 2021). Research has shown that erythromycin, ciprofloxacin hydrochloride, and sulfamethoxazole all have toxic effects on *Selenastrum capricornutum* (Liu et al., 2011; Kiki et al., 2022). The effects of sulfamethazine, enrofloxacin, and ofloxacin on *Scenedesmus obliquus* were all of low toxicity, but all had certain effects on the physiological indexes of *Scenedesmus obliquus* (Chen et al., 2020; Yang et al., 2020b). Studies have found that the toxicity hazard levels of enrofloxacin and ciprofloxacin hydrochloride to *Isochrysis galbana* are high toxicity and poisoning, respectively (Ge and Deng, 2015). Singh et al. (2018) have found that high concentrations of ofloxacin reduced the biomass, relative root growth rate, protein, and photosynthetic pigment content of duckweed, and increased the activity of antioxidant enzymes in leaves. Meanwhile, duckweed also had a significant degradation effect on ofloxacin. Flumequine and enrofloxacin have inhibitory effects on the plant germination, growth and cell division (Migliore et al., 2000). Therefore, antibiotics not only cause oxidative damage to aquatic plants, but also inhibit the growth and metabolism of aquatic plants.

Not only does duckweed provide food and a habitat for other organisms in the aquatic system, but it also assists in the removal of numerous pollutants from water bodies (Gomes et al., 2020). To understand the risks and effects of antibiotic residues in plant systems, we investigated the effect of streptomycin on duckweed by evaluating the biomass of duckweed, changes of photosynthetic pigments, antioxidant enzymes and other indicators, as well as effects on gene transcription and expression. The findings suggested that the antibiotic had a significant effect on duckweed's growth, enzyme content, and metabolic pathways when given a high dose of streptomycin; meanwhile, duckweed made greatly contribute to the streptomycin degradation. In conclusion, as one of the producers in the natural ecosystem, aquatic plants play an important role in rebuilding and restoring the aquatic ecological environment. This study, as a case study, can serve as a theoretical foundation for the ecological risk assessment of emerging pollutants in the water environment.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: NCBI, PRJNA896651.

Author contributions

Conceptualization, WH and YA. Methodology, WH. Investigation, WH, RK and LC. Resources, WH and RK. Data curation, RK. Writing—original draft preparation, WH. Writing—review and editing, WH, LC and YA. Visualization, LC. Project administration, YA. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1065199/full#supplementary-material

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EDITED BY
Pankaj Bhatt,
Purdue University, United States

REVIEWED BY
Junaid Ali Siddiqui,
Guizhou University, China
Deep Chandra Suyal,
Eternal University, India

*CORRESPONDENCE
Xiaolong Hou
xl.hou@fafu.edu.cn

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Transcriptomics-based analysis of genes related to lead stress and their expression in the roots of *Pogonatherum crinitum*

Chenlu Zhu[1], Junbao Yu[1], Shuyi Cao[1], Xinyi Wu[1], Weicai Meng[1] and Xiaolong Hou[1,2,3]*

[1]Institute of Forestry and Environment, College of Forestry, Fujian Agriculture and Forestry University, Fuzhou, China, [2]Key Laboratory of State Administration of Forestry and Grassland on Soil and Water Conservation of Red Soil Region in Southern China, National Forestry and Grassland Administration, Fuzhou, China, [3]Cross-Strait Collaborative Innovation Center of Soil and Water Conservation, Department of Education of Fujian Province, Fuzhou, China

Revealing plants' tolerance and transport genes to heavy metal stress play an important role in exploring the potential of phytoremediation. Taking the heavy metal lead (Pb) hyperaccumulator plant *Pogonatherum crinitum* (Thunb.) Kunth as the research object, a hydroponic simulation stress experiment was set up to determine the physiological indicators such as antioxidant enzymes and non-enzymatic antioxidants in the roots of *P. crinitum* under different Pb concentrations (0, 300, 500, 1000, 2000 mg·L^{-1}). RNA-Seq was performed, the Unigenes obtained by transcriptome sequencing were enriched and annotated by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, and the differential expression genes (DEGs) of root were screened and verified by quantitative real-time polymerase chain reaction (qRT-PCR). The results are as follows: with the increase of Pb concentration, superoxide dismutase (SOD), catalase (CAT), and ascorbic acid (AsA) content increased. Peroxidase (POD), malondialdehyde (MDA), and ascorbic acid–glutathione (AsA-GSH) cycles showed low promotion with high inhibition. A total of 38.21 Gb of bases were obtained by transcriptome sequencing, and the base quality of each sample reached Q20 and Q30, accounting for 90%, making the sequencing results reliable. Combined with transcriptome sequencing, functional annotation, and qRT-PCR validation results, 17 root Pb-tolerant genes of *P. crinitum* were screened out, which were related to antioxidation, transportation, and transcription functions. Moreover, qRT-PCR verification results under different Pb stress concentrations were consistent with the transcriptome sequencing results and changes in physiological indicators. In brief, the root of *P. crinitum* can adapt to the Pb stress environment by up-regulating the expression of related genes to regulate the physiological characteristics.

KEYWORDS

hyperaccumulator, *Pogonatherum crinitum*, Pb stress, transcriptomics, Pb resistance gene, qRT-PCR

1 Introduction

Heavy metal pollution is one of the major problems in global soil pollution, posing a threat to the growth of plants, sustainable development, and human health (Huang and Luo, 2022). Therefore, the problem of heavy metal pollution in soil has attracted the attention of many scholars worldwide. Phytoremediation is a soil pollution remediation technology that integrates the advantages of low cost, high efficiency, and large-scale use with a minimal negative impact on soil structure (Jiang et al., 2019). The ability of plants to enrich heavy metals in soil is the key to phytoremediation technology (Ma et al., 2021). Therefore, studying hyperaccumulators and their physiological responses to heavy metal stress has become a hot topic. However, the wild-type hyperaccumulators found in the literature still have shortcomings regarding environmental impact, growth cycle, and response to species to heavy metals (Chen, 2008); therefore, the research is still in the exploratory stage.

Pogonatherum crinitum (Thunb.) Kunth is a Pb hyperaccumulator plant with large biomass, which can grow normally under Pb stress with a concentration of upto 20,000 $mg{\cdot}kg^{-1}$ (Hou et al., 2019). Under Pb stress, *P. crinitum* can improve the total antioxidant capacity by increasing the content of osmotic regulators (Han et al., 2018) and regulating the ascorbic acid–glutathione (AsA-GSH) cycle (Han et al., 2018) to adapt to the Pb stress environment. It shows that *P. crinitum* resists heavy metal stress in various ways; however, the molecular biology strategy of its response to Pb stress is still unclear.

Various studies have shown that plants may respond to heavy metal stress by regulating genes related to antioxidants, transporters, signal transduction, and transcription factors (Auguy et al., 2013). Plants such as *Trigonella foenum-graecum* L. (Alaraidh et al., 2018), *Medicago Sativa* L. (Lou et al., 2018), and *Brassica juncea* L. (Singh et al., 2020) reduce the toxic effects of heavy metals on plants by regulating the expression of antioxidant enzymes and other related genes to varying degrees. Sun et al. (2019) conducted transcriptome sequencing, enrichment analysis, and verification of the Cadmium (Cd) hyperaccumulator plant *Brassica campestris* L. They found that the up-regulation of key genes in the glutathione (GSH) metabolic pathway is crucial in improving the plant's resistance to Cd stress. *Salvinia minima*, a Pb hyperaccumulator plant, increase the glutamine synthetase SmGS gene's expression level in plants under stress. The subsequent increase in GSH enzyme activity significantly prevents plants from being poisoned by Pb (Neyi et al., 2012). Another study found that Pb induced the FeABCC1 gene to be significantly expressed in the hyperaccumulator plant *Fagopyrum esculentum* Moench and the transformed yeast showed that tolerance to Pb in plants increased significantly with gene expression and accumulated more Pb (Mizuno et al., 2010).

Rini and Hidayati (2021) found that *Saccharum spontaneum* L. stimulated root growth by up-regulating phytochelatins (PCs) gene to adapt to the stress environment under Pb stress. The hyperaccumulator plant *Sedum alfredii* Hance has a strong ability to enrich a variety of heavy metals. In addition, transporter gene SaPCR2 (Ge et al., 2022) and the member of the heat shock transcription factor gene family SaHsfs (Chen et al., 2018) were overexpressed under Pb stress, and these genes may play a role in the detoxification to Pb. The molecular mechanism of Pb tolerance in the hyperaccumulator plant *P. crinitum* is still unclear. Almost no information about genes and their action mechanisms in response to Pb stress leaves a big gap. Therefore this study aims to investigate the response strategies of *P. crinitum* at the molecular level under different Pb stress levels. According to the research objectives, a hydroponic simulation stress experiment with varying concentrations of Pb was used for this study. This study will provide a basis for further revealing the intrinsic mechanism of plant response to Pb stress.

Abbreviations: Ascorbic acid, ASA; Ascorbic acid–glutathione, ASA-GSH; Aseorbate peroxidase, APX; ATP-binding cassette, ABC; Catalase, CAT; Cluster of orthologous groups of proteins, COG; Coat protein II, COP II; Dehydroascorbate, DHA; Dehydroascorbate reductase, DHAR; Differential expression genes, DEGs; False discovery rate, FDR; Gene ontology, GO; Glutathione, GSH; Glutathione oxidized, GSSG; Glutathione peroxidase, GSH-Px; Glutathione reductase, GR; Kyoto encyclopedia of genes and genomes, KEGG; Malondialdehyde, MDA; Metallothioneins, MTs; Monodehydroascorbate, M-DHA; Monodehydroascorbate reductase, M-DHAR; National center for biotechnology information, NCBI; Natural resistance-associated macrophage proteins, Nramp; Nicotinamide adenine dinucleotide, NAD+; Nicotinamide adenine dinucleotide phosphate, NADPH; Non-redundant protein sequence, Nr; Peroxidase, POD; Peroxisomal targeting signal 1, PTS1; Phytochelatins, PCs; Quantitative real-time polymerase chain reaction, qRT-PCR, Reactive oxygen species, ROS; Superoxide dismutase, SOD; v-myb avian myeloblastosis viral oncogene homolog, MYB; Water holding capacity, WHC; ZRT, IRT-like protein, ZIP.

2 Materials and methods

2.1 Experimental material and design

The tested *P. crinitum* was cultivated with seeds. The seeds were collected from the lead-zinc mine in Sanming, Fujian Province. The seeds were spread evenly on the seedbed in the greenhouse to cultivate the plants. Watering was done regularly to keep the soil water holding capacity (WHC) at about 70% for the experiment. *P. crinitum* plants of consistent growth were selected, cleaned in the root system, and transplanted into a hydroponic device with a 1/8 Hoagland nutrient solution. Fifteen plants per pot were transplanted. The Pb stress test

was initiated after cultivating the plants in the nutrient solution for three days.

Two experiments were set up: one group at 1000 mg·L^{-1} Pb stress treatment (indicated by TS in the text) and a control experiment without Pb stress (indicated by CS in the text). The other group was set up with different concentrations of Pb stress treatment :0, 300, 500, 1000, 2000 mg·L^{-1} (represented by CK, Pb300, Pb500, Pb1000, and Pb2000 in the text). Using the 60 mg·L^{-1} Pb solution prepared by $(CH_3COOH)_2Pb$, the corresponding Pb solution is added to the culture device with the nutrient solution according to the designed stress concentrations. The nutrient solution components are shown in supplementary material; the concentration of KH_2PO_4 was reduced from 0.136 g·L^{-1} in the original formula to 0.68 mg·L^{-1} to avoid precipitation (Table S1).

After repeated washing with deionized water, the roots of the *P. crinitum* were moved into the stress solution of different Pb concentrations and placed in an artificially controlled incubator for the experiment (conditions were 25°C, 75% humidity, 6000 Lx light intensity, and day and night light time was (16/8) h·d^{-1}), and the stress time was 7 d. To ensure that *P. crinitum* can grow normally under hydroponic conditions, hydroponic pots were aerated twice every morning and evening for 10 min and supplemented with the nutrient solution without Pb to the original nutrient solution scale. Three repetitions were used for each treatment.

After the stress experiment, harvested fresh *P. crinitum* plants were washed repeatedly with deionized water and dried adequately with filter paper (It takes about 52 d from seed to harvest fresh *P. crinitum* sample, of which 45 d was required for plants grown from seed to 15 cm and 7 d for hydroponic stress experiment). The fresh root samples were quick-frozen in liquid nitrogen and placed in a -80°C refrigerator for later use. The CS and TS-treated samples were used for high-throughput transcriptome sequencing. The samples treated with CK, Pb300, Pb500, Pb1000 and Pb2000 were used for physiological indicators, RNA extraction and fluorescence quantification. In addition, each treatment was repeated three times.

2.2 Experiment methods

2.2.1 Physiological indexes measurement

To prepare the enzymatic solution, 0.2 g of fresh roots were weighed and ground into a homogenate in a mortar with liquid nitrogen; then, put into a 4 mL centrifuge tube; added 1 mL of 0.05 mol·L^{-1} phosphate buffer at pH 7.8, and then fix the volume to 4 mL. The solution was mixed well with a vortexer, then centrifuged in a refrigerated high-speed centrifuge at 4°C with 10,000 rpm for 10 min. The supernatant was taken and put in the 4°C refrigerators for later use. The activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and malondialdehyde (MDA) were determined by nitrogen blue tetrazolium, ultraviolet absorption, guaiacol, and thiobarbituric acid method, respectively (Zou, 1995). For GSH and ascorbic acid (ASA) determination methods, we used methods mentioned by Ma and Cheng (2003) and Tanaka et al. (1985), respectively. The APX activity was consistent with Nakano and Asada (1981).

2.2.2 RNA extraction, sequencing, and assembly

The root RNA was extracted using the RNA prep Pure polysaccharide and polyphenol plant total RNA extraction kit produced by TIANGEN. The concentration and purity of RNA were detected by ultra-micro spectrophotometer (DeNovix Company, DS-11+Spectrophotometer, the concentration should be greater than 50 ng·μl^{-1}). OD values were between 1.8-2.0 to ensure the purity of RNA). 1% agarose gel was prepared; electrophoresis was for about 20 min. The gel was placed on an automatic gel imager to observe the sample's integrity. After quality inspection of the obtained RNA, a library was established and sequenced using the Illumina Hi Seq 4000 sequencing platform. The resulting clean reads were assembled and evaluated using Trinity (Grabherr et al., 2011). RSEM quantifies the assembled Unigenes.

2.2.3 Functional annotation of Unigenes and differential expression genes analysis

Assembled Unigenes were aligned with non-redundant protein sequence (Nr), a cluster of orthologous groups of proteins (COG), gene ontology (GO), Kyoto encyclopedia of genes and genomes (KEGG), and SwissProt databases and highly similar proteins to annotate the IDs of the Unigenes. The reads count data in the gene expression level were analyzed using DESeq2 to obtain differential genes (Love et al., 2014). Genes with false discovery rate (FDR) < 0.05 and |log2FC| >1 were considered significantly different expression genes. The Blast2go software was used to compare the significantly different Unigenes with the KEGG and GO databases for annotation and enrichment analysis and combined with the functional annotation and pathway enrichment results to screen candidate genes related to Pb resistance.

2.2.4 Reverse transcription and quantitative real-time PCR

The cDNA was obtained by reverse transcription using the Uni All-in-one First-Strand cDNA Synthesis SuperMix for qPCR Reverse Transcription Kit produced by Transgen. The selected primers for Pb-resistant genes were designed using the premier designing tools in National Center for Biotechnology Information (NCBI). Then DNAMAN 8, PCR amplification, and electrophoresis verified the stability of each primer. The primers of the Pb-tolerant gene in the roots of *P. crinitum* are shown in the supplementary materials (Table S2). qRT-PCR was

performed using the QuanStudio3 system fluorescence quantitative PCR instrument of Fuzhou Dobiotech. The reaction system was: 0.4 μL of upstream and downstream primers (10 μM), 1 μL of cDNA, 10 μL of 2×PerfectStart Green qPCR SuperMix, and Nuclease-free Water 8.2 μL for a total of 20 μL. The reaction process was 94°C for 30 s; 94°C for 5 s; 60°C for 30 s, a total of 40 cycles. Each sample has three biological replicates, and each has three technical replicates, and the relative expression levels of genes were calculated using $2^{-\Delta\Delta Ct}$.

2.3 Statistical analysis

Using SPSS 25 software, one-way analysis of variance (One-way ANOVA) and Tukey's *post-hoc* test were used for multiple comparisons of experimental data and followed by multiple comparisons using the least significant difference (LSD) test. The level of significance was set at $P < 0.05$ (two-tailed). All the test results are expressed as mean ± standard deviation. The differential gene volcano map, heat map, GO entry enrichment map, and KEGG pathway enrichment map are all drawn using the cloud tools of Omicshare. The dynamic heat map of Omicshare and Adobe Illustrator CC jointly drew the pathway analysis map. The relative expression histogram is drawn using Origin 2017.

3 Results

3.1 Physiological indexes under different Pb stress

Data in Table 1 show that, under Pb stress, SOD, CAT, and ASA content showed an upward trend with the increase of Pb concentration, and the differences among the treatments were greater than those of CK ($P < 0.05$). SOD and CAT treated with Pb2000 increased by 24.53% and 66.34%, respectively, compared with CK. The contents of POD, aseorbate peroxidase (APX), and GSH in roots reached were higher when treated with Pb500 and increased by 14.68%, 444.22%, and 30.37%, respectively, compared with the control. When the concentration of Pb was less than 1000 $mg{\cdot}kg^{-1}$, the MDA content increased with the concentration increase. The effects of different Pb stress on these indicators are low promotion and high inhibition.

3.2 Transcriptome sequencing analysis under Pb stress

3.2.1 Sequencing results

Two groups of samples were assembled and filtered to obtain a total of 38.21 Gb of total bases, the GC ratio was 52.71%-53.14%, and the base composition was balanced. The number of bases with base quality values above Q20 in each sample account for more than 95%, and those with base quality above Q30 were more than 90%, indicating that each sample's sequencing quality is high and meets the requirements for library construction (Table S3).

3.2.2 Analysis of DEGs

Based on the different analysis results, genes with FDR < 0.05 and |log2FC| >1 were screened as significantly different genes (Figure 1A). There were 51,281 unigenes with significant differences ($P < 0.05$), of which 21,138 were up-regulated (accounting for 41.22%), and 30,143 were down-regulated. Figure 1B shows that the genes related to antioxidant enzymes were up-regulated, consistent with the response trend of physiological indicators in roots under Pb stress. In addition, the expression levels of genes related to heavy metal transport [ATP-binding cassette (ABC), natural resistance-associated macrophage proteins (Nramp), metallothioneins (MTs)], transcription factor genes (WRKY, NAC), ATPase, GTP-binding proteins, heat shock proteins, and disease course-related proteins (Unigene47646_All) were increased. However, genes related to cell wall proteins (cell wall protein, *CL9150.Contig2_All*, *Unigene48447_All*) and genes related to plant hydrolysis and metabolism (caspases, calcium-binding

TABLE 1 The physiological characteristics in the roots of *P. crinitum* under different Pb stress concentrations.

Physiological indicators	Treatment				
	CK	Pb300	Pb500	Pb1000	Pb2000
SOD activity	1832.42 ± 46.11d	2170.04 ± 56.84b	2367.1 ± 82.14a	1971.36 ± 123.14c	2281.86 ± 62.79ab
POD activity	341.72 ± 3.90c	367.70 ± 18.76b	391.88 ± 3.26a	369.74 ± 11.85b	374.20 ± 8.19b
CAT activity	34.69 ± 2.05c	40.05 ± 3.21c	48.78 ± 4.07b	51.38 ± 3.52ab	57.71 ± 2.95a
MDA content	10.63 ± 1.47c	11.57 ± 1.55bc	13.81 ± 2.21b	26.13 ± 5.11a	11.4 ± 1.67bc
APX activity	0.09 ± 0.01b	0.1 ± 0.01b	0.48 ± 0.1a	0.17 ± 0.04b	0.14 ± 0.03b
ASA content	5.02 ± 0.49c	6.13 ± 0.40b	5.03 ± 0.19c	5.44 ± 0.26c	6.69 ± 0.65a
GSH content	1.22 ± 0.04d	1.36 ± 0.06b	1.59 ± 0.04a	1.27 ± 0.04cd	1.29 ± 0.04c

Different lowercase letters in the same row represent significant differences among different treatments ($P < 0.05$).

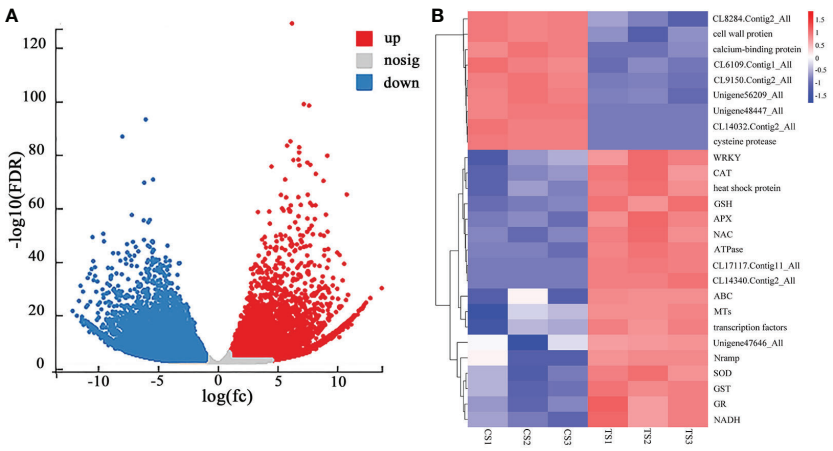

FIGURE 1
(A) CS-vs-TS difference volcano map of roots, and **(B)** heat map of the expression of some Pb-tolerant DEGs in roots of *P. crinitum*. (1000 mg·L^{-1} Pb stress treatment is indicated by TS and control experiment without Pb stress is indicated by CS).

proteins, *CL14032.Contig2_All*, *Unigene56209_All*, *CL6109.Contig1_All*) showed down-regulated expression. This may be because Pb stress damages the cell wall and some mechanisms related to plant growth and metabolism, hindering its expression.

3.2.3 GO enrichment analysis of DEGs

By comparing with the GO database, the differential genes in the roots of *P. crinitum* under Pb treatment are annotated to 44 GO terms, divided into three categories: biological process, cell component, and molecular function. The main functional items in the biological process were the metabolic process and regulation. In the molecular function, the entries with the most significant number of DEGs were binding, catalytic activity, and transporter activity. In the cellular component, the most enriched number was the cellular anatomical entity. It indicates that the root system of *P. crinitum* under Pb stress might adapt to the Pb stress environment through metabolism, generation of new substances, catalytic enzyme activity, and transport of heavy metals (Figure 2).

As shown in Table S4, a total of 8447 DEGs were significantly enriched in 42 secondary entries related to Pb resistance, including antioxidant enzymes, transport, transcription, ubiquitin, and signaling ($P < 0.05$). The complete details are shown in (Table S4). Among them, 1773 DEGs and 1677 DEGs were enriched in antioxidant-related and transcription-related GO items, respectively. Among the significantly enriched entries, four items are related to transport, accounting for 9.53%. The GO items related to signaling account for 6.12%, the DEGs enriched in the GO items related to protein kinases were the most, and the genes significantly enriched in the GO term "protein kinase activity" account for 2.13%.

3.2.4 KEGG enrichment analysis of DEGs

Annotation and enrichment analysis are carried out by comparing the DEGs in the KEGG pathway database's root system. A total of 15,291 differential genes were significantly enriched in 25 pathways ($P < 0.05$). DEGs on the "RNA transport" pathway were the most significant and enriched, with a total of 2983, accounting for 19.51% of the total DEGs significantly enriched in the KEGG database (Figure 3).

The root differential genes were mainly enriched in "Phenylpropanoid biosynthesis," "MAPK signaling pathway - plant," and "Plant hormone signal transduction," accounting for 8.96%, 6.29%, and 5.48%, respectively. Among the KEGG pathways significantly enriched in DEGs, "Flavonoid biosynthesis," "Ascorbate and aldarate metabolism", "Isoflavonoid biosynthesis," "Plant-pathogen interaction" and "Cutin, suberine, and wax biosynthesis," were all related to plant resistance to heavy metals related, accounting for 49.87% of the total DEGs significantly enriched in the KEGG database. These results indicated that the root system of *P. crinitum* may respond to Pb stress mainly through the transport system. At the same time, signal transmission, antioxidant enzyme, and disease resistance systems also respond to Pb stress. Moreover, KEGG pathways such as "Flavone and flavonol biosynthesis," "Alanine, aspartate and glutamate metabolism," "Phenylalanine metabolism," "Glutathione metabolism," "ABC transporters," "Basal transcription factors," and "Peroxisome" are all related to Pb resistance. A total of 2507 DEGs were enriched, accounting for 7.06% of all differential genes enriched in the KEGG database.

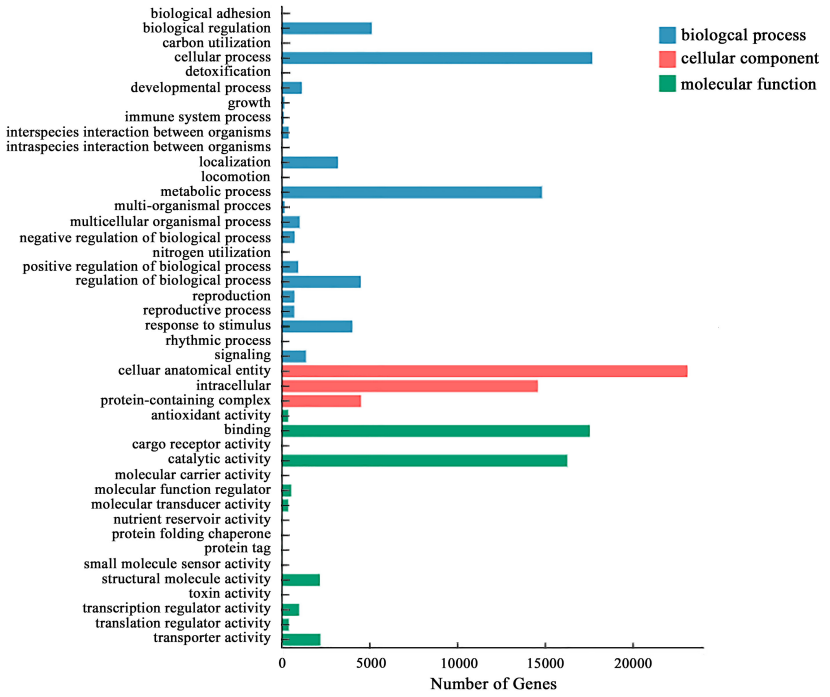

FIGURE 2
Gene ontology (GO) enrichment classification map of differentially expressed genes in the roots of *P. crinitum* under Pb Stress.

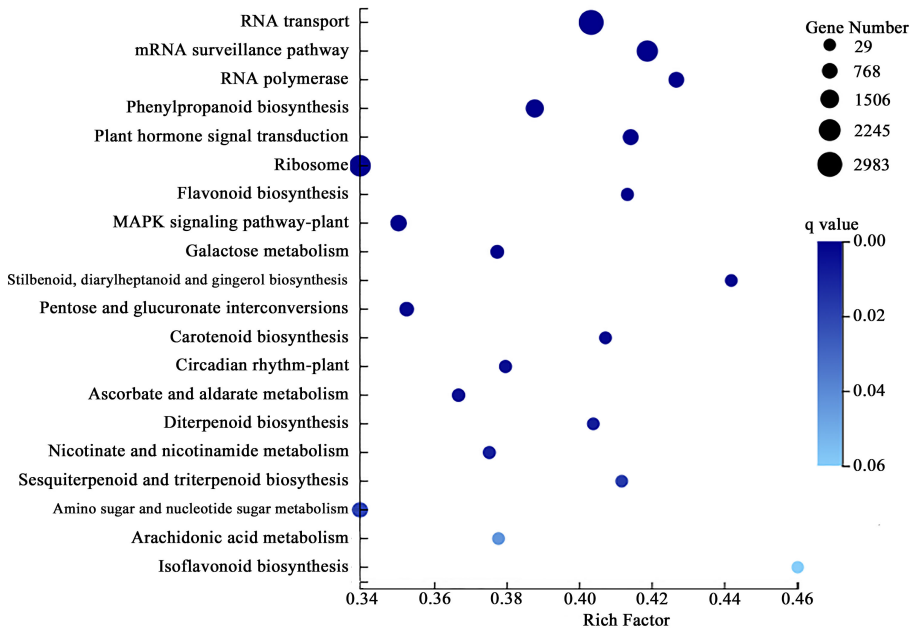

FIGURE 3
Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis of differential expression genes (DEGs) in the roots of *P. crinitum* under Pb Stress.

3.3 Pathway analysis of Pb-tolerant candidate genes in roots of *P. crinitum*

Combined with the expression and functional annotation results of differential genes, DEGs with significant differences in Pb tolerance were screened from the GO items and significantly enriched KEGG pathways. The genes related to root antioxidants were: *CL17117.Contig11*, *CL17117.Contig17* and *CL762.Contig2* was all involved in the "Peroxisome" pathway, which belongs to the peroxisome targeting signal (PTS1 type) in the antioxidant enzyme system.

As shown in Figure 4, there were 5 DEGs associated with the ASA-GSH cycle:and all acted on Glutathione metabolism. Among them, *CL871.Contig1* and *CL5100.Contig1* were located at "1.11.1.11" and "1.6.5.4", respectively, and both sides were L-Ascorbate and Monodehydro-ascorbate, but the two work in the opposite direction. It indicates that these two genes acted on the redox of ascorbic acid in the roots of *P. crinitum*. *CL12071.Contig1* was located at "1.8.1.7" and acts on the transition from GSSG to GSH, while *CL13989.Contig3* was located at "1.11.1.9" and acts on GSH to GSSG. *CL2332.Contig13* was located in "2.5.1.18", and the back end of this gene is meicaptuiac acid, indicating that this gene may be involved in converting glutathione to meicaptuiac acid, thereby adapting to the Pb stress environment.

A total of 4 root-Pb tolerance genes related to transport were screened: *CL1174.Contig14* participates in "ABC transporters" and is located as an ABC-B class transporter, *CL4795.Contig9*, *CL12532.Contig3* and *CL14340.Contig2* were annotated as a natural resistance-associated macrophage protein, mitochondrial import inner membrane translocase subunit, and COPII protein in the "Protein processing in endoplasmic reticulum" pathway, respectively, indicating that roots under Pb stress might alleviate the toxicity through the differential expression of these transporter-related genes.

Furthermore, five root DEGs were also screened to be related to plant signal transduction and transcription under Pb stress: *Unigene46823* was involved in "Plant-pathogen interaction", located in "WRKY", and played a role in the induction of defense-related genes to resist abiotic stress. *CL8121.Contig3* was involved in the "MAPK signaling pathway - plant," a copper ion exporting ATP enzyme, and acted on the defense response pathway of the plant hormone Ethylene. *CL14444.Contig1* was involved in "1.11.1.7" in "Phenylpropanoid biosynthesis" in Figure 4 as the activation gene of heat shock protein ATPase, and the upstream and downstream were aldehyde and lignin, respectively, indicating that this gene might be involved in the conversion of aldehydes into lignin, thus responding to the Pb stress environment. *CL1583.Contig2* was annotated as a two-component system protein gene of the "Plant hormone signal

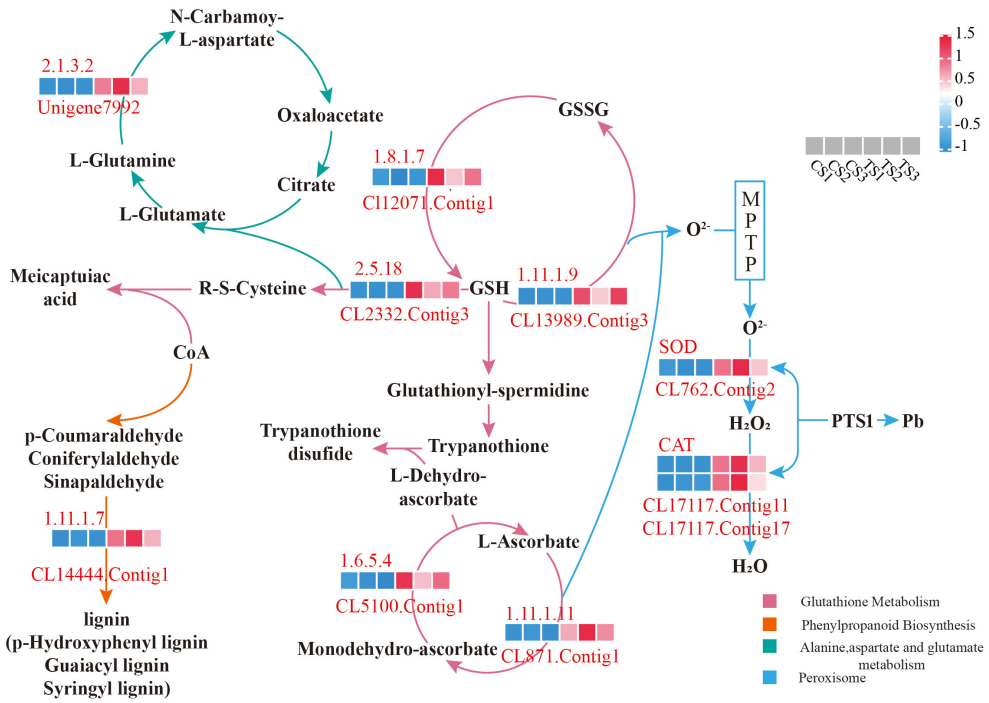

FIGURE 4
Partial possible molecular mechanism of Pb tolerance in roots of *P. crinitum*.

transduction" pathway. As shown in Figure 4, *Unigene7992* participates in the "Alanine, aspartate and glutamate metabolism" pathway, which was located in the process of "2.1.3.2" acting on the conversion of L-Glutamine to L-Aspartate, and the pathway belonged to the conversion of L-Glutamate.

3.4 qRT-PCR validation and analysis of Pb-tolerant candidate genes

The expression levels of the selected DEGs were analyzed to verify the transcriptome sequencing results and Pb-tolerant candidate genes in roots. As shown in Figure 5A, the relative expression of *CL17117.Contig17* increases with the increase of Pb concentration, and the difference between treatments was significant ($P < 0.05$). Under the treatments of different Pb concentrations (Figure 5D), the relative expression levels of *CL8121.Contig3* were significantly higher than those in control ($P < 0.05$), and the expression under Pb2000 treatment was significantly higher than that in other treatments ($P < 0.05$), compared with Pb1000 treatment, the up-regulation degree was increased by 55.52%. In addition to Pb300, the relative expression levels of *CL13989.Contig3*, *CL5100.Contig1*, *CL2332.Contig13* (Figure 5B), *CL12532.Contig3*, *CL14340.Contig2* (Figure 5C) and *Unigene7992*, *CL14444.Contig1* (Figure 5D) under each treatment was significantly greater than those of CK ($P<0.05$). The gene expression under the Pb2000 treatment was 7.47, 10.28, 3.52, 5.75, 73.36, 20.16, and 12.65 times that of the control.

The relative expression levels of *CL17117.Contig11*, *CL762.Contig2* (Figure 5A), *CL871.Contig1*, *CL12071.Contig1* (Figure 5B), *CL1583.Contig2* and *Unigene46823* were significantly higher than CK under Pb1000 and Pb2000 treatments ($P < 0.05$). The expression levels of these genes under Pb1000 treatment are increased by 48.21%, 892.11%, 341.28%, 142.37%, 183.97%, 180.51% and 207.94% compared with CK, respectively. Comparing Pb1000 with Pb2000 treatment, the expression levels were increased by 90.72%, 97.05%, 51.02%, 62.67%, 60.57%, 56.61% and 99.55%, respectively. The relative expression of *CL4795.Contig9* at the Pb concentration of 1000 mg·L^{-1} was significantly higher than that of CK ($P < 0.05$), which is 1.52 times that of the control (Figure 5C).

4 Discussion and conclusions

The main way for plants to deal with Pb toxicity is to improve their antioxidant capacity (Song et al., 2015). In this study, with the increase of Pb concentration, the activities of POD, SOD and CAT enzymes in the roots of *P. crinitum* generally showed an upward trend. This is because Pb stress activates antioxidant enzymes in the roots, and by increasing their activity, it can remove excess reactive oxygen species (ROS) in the plant and improve the antioxidant capacity of plants to improve the Pb tolerance. *Iris ensata* (Bo et al., 2021), *Brassica juncea* (Małecka et al., 2019), *Medicago sativa* (Helaoui et al., 2020) species have similar response mechanisms. In this study, the content of MDA in roots of *P. crinitum* increased with the increase of Pb stress, indicating that Pb stress caused damage to root membrane lipidation and stimulated the antioxidant system. At Pb2000, the MDA content was significantly lower than other treatments, which is because the antioxidant enzymes in the plants scavenge excess ROS, and the antioxidant defense system alleviates Pb toxicity (Cedeno and Swader, 1972; Mao et al., 2019).

In this study, the GSH content and APX enzyme activity in the roots of *P. crinitum* showed an increasing trend with the concentration increase. This is because the reactive oxygen system was out of balance under Pb stress, and GSH and ASA undergo a reduction reaction with ROS, thereby maintaining dynamic balance (Wang et al., 2021). APX participates in the ASA-GSH cycle as a coenzyme and acts together with POD and CAT to decompose H_2O_2 (Qin et al., 2018). When the concentration is more significant than 500 mg·L^{-1}, the non-enzymatic antioxidants in *P. crinitum* decreased with the concentration increase. This may be because the high concentration of Pb inhibits the enzymatic reaction in the GSH-ASA cycle, and the synthesis of GSH and ASA is hindered (Liu et al., 2019).

In this study, Pb-resistant candidate genes were screened by transcription sequencing of the roots of TS and CS. GO enrichment analysis found that the most significant GO entry for root DEGs was the metabolic process, followed by a catalytic activity. One of the main ways in which plants respond to external stimuli is metabolite regulation, which can resist abiotic stress by metabolizing or generating secondary metabolites (Sarvajeet and Narendra, 2010). The DEGs in the root system of *P. crinitum* were significantly enriched in GO items because plants produce a large amount of ROS under abiotic stress, and scavenging excess ROS is the main way for plants to adapt to stress and toxicity (Lu et al., 2020). In addition, root DEGs are also significantly enriched on nicotinamide adenine dinucleotide phosphate (NADPH)-related terms. Under Pb stress, antioxidant-related genes in roots promote nicotinamide adenine dinucleotide (NAD+) generation to ensure the reduction reaction of NADPH and inhibit the generation of reactive oxygen species (Zhang et al., 2014). In addition to antioxidant-related GO terms, root DEGs were significantly enriched in functional terms related to transcription, transport, and protein kinases. The possible reason is that the root system is an organ with a high degree of Pb enrichment. Under stress, many gene-regulated plants can improve stress resistance in different ways, which is consistent with Yuan's research results (Yuan et al., 2017). DEGs in roots of

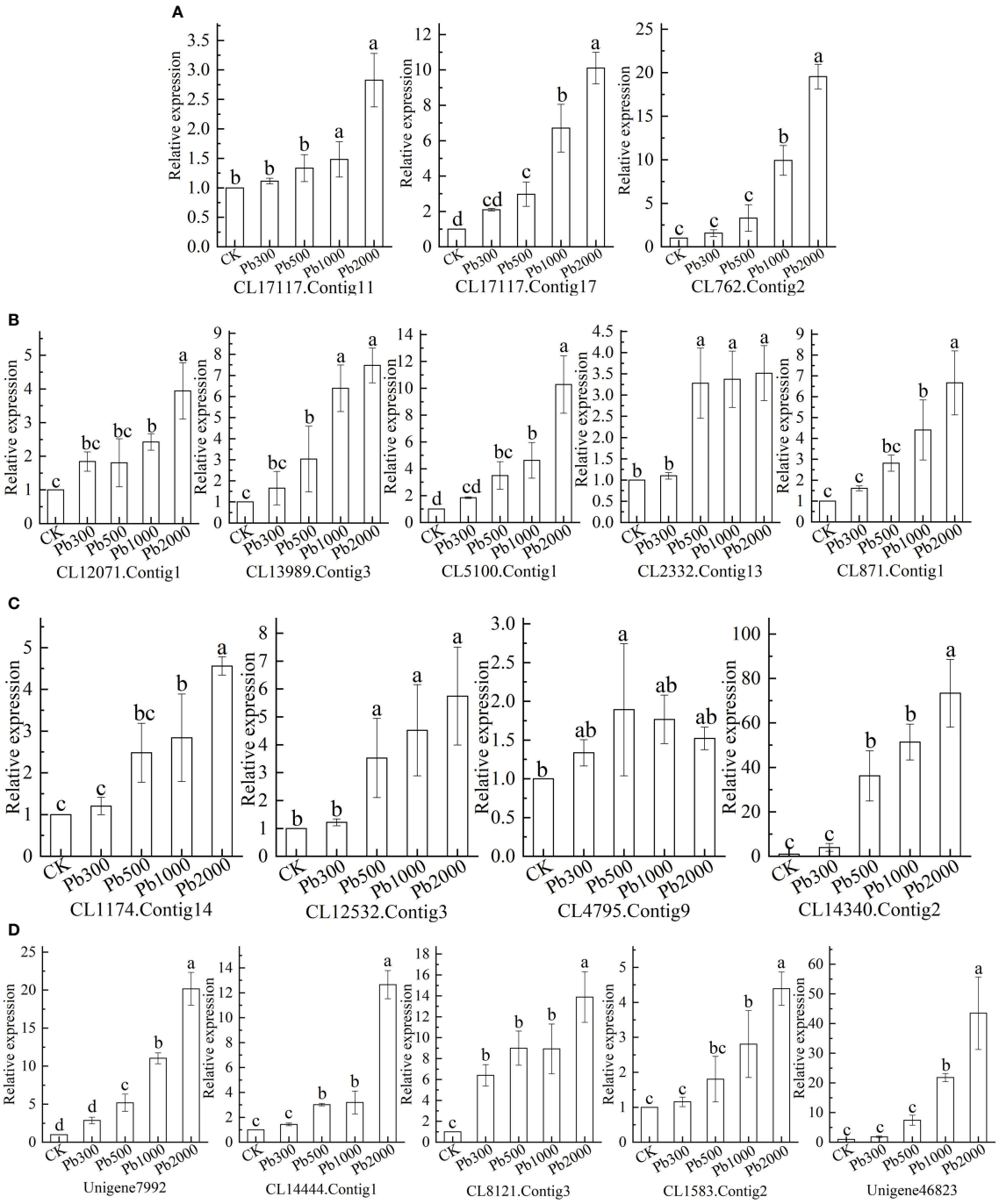

FIGURE 5
The expression of **(A)** antioxidant-related genes, **(B)** Ascorbic acid–glutathione (ASA-GSH) cycle-related genes, **(C)** transporter-related genes, and **(D)** signal transduction and transcription-related genes in the roots of *P. crinitum* under different lead stress concentrations.

P. crinitum were significantly enriched in Pb tolerance-related pathways such as "Phenylalanine metabolism" and "Phenylpropane biosynthesis" under Pb stress. This is because phenolic compounds such as flavonoids and lignin, as the products of phenylpropane metabolism, mainly play a role in removing excess ROS in plants and improving plant antioxidants (Xie and Zhang, 2003), and phenylalanine is involved in this metabolic process as the main ammonia enzyme (Ouyang and Xue, 1988); therefore, the roots of *P. crinitum* can promote phenylpropane biosynthesis through gene

regulation and improve its tolerance to Pb stress, which is consistent with the response of the phenylalaninase gene of *Oryza sativa* L. subsp. indica to heavy metal stress (Huang et al., 2012). In addition to antioxidant and transport-related KEGG pathways, root differential genes were significantly enriched in signal transduction-related pathways such as "MAPK signaling pathway-plant" and "Plant hormone signal transduction". This is because signal transduction is activated by the inducement of an abiotic stress environment, in which ROS and plant hormones generated by stress are essential components in the signal transduction process (Wang et al., 2012). Besides, DEGs are also significantly enriched in pathways such as "Flavonoid biosynthesis" and "Ascorbic acid and aldehyde salt metabolism".

In this study, *CL17117.Contig11*, *CL17117.Contig17* and *CL762.Contig2* in the "peroxisome" pathway were up-regulated expressed, the peroxisomal targeting signal 1 (PTS1) in the antioxidant system. As an important organelle in response to abiotic stress, peroxisomes cannot generate related enzymes by themselves, and PTS is required to direct enzyme precursors into peroxisomes (Zhao, 2000). Pb stress may stimulate the production of PTS1 by peroxisomes in the roots of *P. crinitum*, promotes the directional transport of ribosomes and the expression of these genes to synthesize antioxidant enzymes such as CAT and SOD, and scavenge O^{2-}. Under Pb stress, *CL14444.Contig1* is up-regulated in the "phenylpropane biosynthesis" pathway as an upstream gene in the lignin synthesis pathway, which is because this gene promotes the conversion of aldehydes to lignin. As an important component of the cell wall, the increase of lignin content means that the root system can improve the stress resistance of plants by inhibiting the production of oxygen free radicals (Ortega and Peragón, 2009) and increasing the strength of the cell wall (Long et al., 2021). This result is consistent with the response strategy of *Pyrus pyrifolia* to abiotic stress (Lu et al., 2015).

In this study, with the increase of Pb stress concentration, the relative expression levels of CAT, SOD, and POD-related genes gradually increase, which is consistent with the changing trend of root antioxidant enzyme activities, indicating that *P. crinitum* may adjust the enzyme activity of the plant through these antioxidant-related genes to adapt it to the Pb stress environment. Studies have shown that under heavy metal stress, antioxidant-related genes in *Vicia faba* L. (Liu et al., 2020), *Triticum aestivum* L. (Navabpour et al., 2020), and *Phytolacca americana* L. (Zhao et al., 2012) plants are also up-regulated and their enzyme activity changes. With the increase of Pb stress concentration, the relative expression levels of the GSH-ASA cycle-related genes are all up-regulated, and all are consistent with the non-enzymatic antioxidant activity. *CL12071.Contig1* and *CL13989.Contig3* are annotated as glutathione reductase (GR) and glutathione peroxidase (GSH-Px), respectively. The up-regulated expression of the two may be because Pb stress induces GR gene expression and promotes the expression of reduced GSSG to GSH. GSH regulates intracellular and extracellular osmotic pressure and chelates with heavy metals (Sousa et al., 2015), reducing Pb toxicity. The expression of GSH-Px further promotes the reaction between GSH and reactive oxygen species to generate glutathione oxidized (GSSG) (Zhao, 2017). Therefore, the GSSG-GSH cycle maintains plants' antioxidant capacity and further improves plants' stress resistance. *CL871.Contig1* and *CL5100.Contig1* were up-regulated with increasing concentration and annotated as APX, and monodehydroascorbate reductase (M-DHAR), respectively. Under Pb stress, *P. crinitum* produced excess ROS and reacted with ASA. The up-regulated expression of the APX gene increases the activity of this enzyme and promotes the oxidation of ASA (Zhou and Mo, 2003). The product of this reaction, monodehydroascorbate (M-DHA) is unstable, and the stress environment stimulates the upregulation of dehydroascorbate reductase (DHAR) gene, which accelerates the reduction of reductase to ASA, thereby further accelerating the scavenging of reactive oxygen species (Li et al., 2007). The oxidation product of M-DHA is dehydroascorbate (DHA), which participates in the metabolic process of spermidine, which can improve the activity of plant antioxidant enzymes and maintain the balance of reactive oxygen species (Liu et al., 2007). *CL2332.Contig13* is expressed on the pathway of GSH conversion to cysteine, which is consistent with the response mechanism of *Porphyra yezoensis* Ueda under Pb stress (Zhou et al., 2011); it is because cysteine cannot only directly react with heavy metals but also reduce the toxicity of heavy metals to plants using transporters (Zhao et al., 2012; Zhang et al., 2017; Abdulla et al., 2019).

The natural resistant macrophage protein gene *CL4795.Contig9* was also up-regulated by Pb stress induction. It is because the protein is tissue-specific and plays a decisive role in the uptake of Pb in the root system of *P. crinitum* (Liu et al., 2022). The root *CL14340.Contig2* belongs to the coat protein II (COP II) in the endoplasmic reticulum, and its expression is up-regulated under Pb stress, which promotes the transport of COP II vesicles to proteins which respond to Pb stress (Zhang et al., 2020). Genes related to transcription factors such as WRKY, v-myb avian myeloblastosis viral oncogene homolog (MYB), and ZRT, IRT-like protein (ZIP) were highly expressed under Pb stress. The possible reason is Pb stimulates the expression of these transcriptomic factors through signal transduction and responds positively to stress, thereby inducing the synthesis of enzymes related to defense against stress and further improving plant stress resistance (Yang et al., 2020). *CL8121.Contig3* acts on the ethylene response pathway, and Pb stress stimulates the expression of this gene, thereby promoting plant ethylene response to the stress environment. Ethylene metabolism can promote the synthesis of antioxidant enzymes and cell lignification to improve plant cell defense capacity (Lu et al., 2020). The two-component system is one of the most important systems in plant hormone signaling, *CL1583.Contig2* regulates the regulator in this system, and Pb stress stimulates the expression of this gene, transmits the signal received by the sensor, and induces the expression of tolerance genes (Lei et al.,

2004). Pb stress induces up-regulated expression of *Unigene7992* in the "Alanine, aspartate and glutamate metabolism" pathway, which may be because the gene promotes the synthesis of aspartate and interacts with other proteins to participate in the stress response (Wu et al., 2013). At the same time, its subsequent products, oxaloacetic acid, and citric acid, not only improve the activity of antioxidant enzymes (Basit et al., 2021) but also react with the product glutamic acid and heavy metals, reducing the effectiveness of Pb elements in plants (Guo et al., 2017).

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/sra/PRJNA887529.

Author contributions

CZ performed the experiments, carried out most data analysis, and wrote the manuscript. JY, SC, XW, WM and XH advised on the experiments and assisted with experiments. XH guided the entire experiment and corrected the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1066329/full#supplementary-material

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EDITED BY
Muhammad Musa Khan,
Zhejiang University, China

REVIEWED BY
Taimoor Hassan Farooq,
Bangor College China, China
Muhammad Azhar Hussain,
Hainan University, China

*CORRESPONDENCE
Xiong Li
lixiong@mail.kib.ac.cn

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Rhizospheric microbiomics integrated with plant transcriptomics provides insight into the Cd response mechanisms of the newly identified Cd accumulator *Dahlia pinnata*

Xiong Li[1,2,3]*, Boqun Li[4], Tao Jin[4], Huafang Chen[1,2], Gaojuan Zhao[1,2], Xiangshi Qin[3], Yongping Yang[3,5] and Jianchu Xu[1,2]

[1]Department of Economic Plants and Biotechnology, Yunnan Key Laboratory for Wild Plant Resources, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China, [2]Center for Mountain Futures, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China, [3]Germplasm Bank of Wild Species, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China, [4]Science and Technology Information Center, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China, [5]Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Xishuangbanna, China

Phytoremediation that depends on excellent plant resources and effective enhancing measures is important for remediating heavy metal-contaminated soils. This study investigated the cadmium (Cd) tolerance and accumulation characteristics of *Dahlia pinnata* Cav. to evaluate its Cd phytoremediation potential. Testing in soils spiked with 5–45 mg kg^{-1} Cd showed that *D. pinnata* has a strong Cd tolerance capacity and appreciable shoot Cd bioconcentration factors (0.80–1.32) and translocation factors (0.81–1.59), indicating that *D. pinnata* can be defined as a Cd accumulator. In the rhizosphere, Cd stress (45 mg kg^{-1} Cd) did not change the soil physicochemical properties but influenced the bacterial community composition compared to control conditions. Notably, the increased abundance of the bacterial phylum Patescibacteria and the dominance of several Cd-tolerant plant growth–promoting rhizobacteria (e.g., *Sphingomonas*, *Gemmatimonas*, *Bryobacter*, *Flavisolibacter*, *Nocardioides*, and *Bradyrhizobium*) likely facilitated Cd tolerance and accumulation in *D. pinnata*. Comparative transcriptomic analysis showed that Cd significantly induced ($P < 0.001$) the expression of genes involved in lignin synthesis in *D. pinnata* roots and leaves, which are likely to fix Cd^{2+} to the cell wall and inhibit Cd entry into the cytoplasm. Moreover, Cd induced a sophisticated signal transduction network that initiated detoxification processes in roots as well as

ethylene synthesis from methionine metabolism to regulate Cd responses in leaves. This study suggests that *D. pinnata* can be potentially used for phytoextraction and improves our understanding of Cd-response mechanisms in plants from rhizospheric and molecular perspectives.

KEYWORDS

heavy metal contamination, phytoextraction, *Dahlia pinnata*, rhizobacteria, signal transduction

1 Introduction

Cadmium (Cd) is a toxic soil contaminant worldwide (Fan et al., 2022) and preventing it from entering the human body from the environment *via* the food chain (Mao et al., 2022) is a great challenge. Many techniques have been developed to minimize Cd absorption and accumulation in crops (Rizwan et al., 2016; Liu et al., 2018; Shi et al., 2020), but such measures can only temporarily address the problem. The long-term solution is to remove Cd from contaminated soils.

Phytoextraction is a sustainable remediation strategy that removes soil Cd by harvesting the aboveground parts of Cd (hyper)accumulators (Oladoye et al., 2022). Although many potential Cd (hyper)accumulators have been screened from naturally or artificially Cd-contaminated soils (Reeves et al., 2018), their widespread application is restricted by insufficient biomass accumulation, Cd bearing capacity, and environmental adaptability (Shen et al., 2021). Thus, further screening and molecular-assisted breeding are needed to identify or produce ideal Cd phytoextractors. Alternatively, uncovering the mechanisms of rhizospheric effects (Khanna et al., 2019) on plant responses to Cd will also benefit the development of feasible strategies that enhance phytoextraction efficiency.

The largest angiosperm family, Asteraceae, has served as an important source of Cd (hyper)accumulators (Nikolic and Stevovic, 2015; Reeves et al., 2018). *Dahlia pinnata* Cav., a perennial herb of Asteraceae with a large biomass (Supplementary Figure S1), is one of the most widely cultivated ornamental plants worldwide (Liu et al., 2010). The wide cultivation of this species implies its strong adaptability to diverse environments. These characteristics indicate its natural advantages in the phytoremediation of heavy metal-contaminated soils. To date, the accumulation characteristics of chromium, lead, and arsenic in *D. pinnata* have been explored (Ramana et al., 2013; Cui et al., 2014; Raza et al., 2019), but few data are available on the Cd tolerance and accumulation of this species.

In this study, we analyze the Cd tolerance and accumulation capacity of *D. pinnata* while determining how its rhizospheric microenvironments (especially rhizobacteria) varied under Cd stress. Additionally, we performed transcriptomic analysis to explore the molecular underpinnings of the Cd stress response in *D. pinnata*. This study will improve our understanding of the Cd response mechanisms in plants from rhizospheric and molecular perspectives.

2 Materials and methods

2.1 Experimental design

2.1.1 Pot experiments

The soils used for a previous study (Li X. et al., 2022) were sieved and mixed to obtain homogeneous composite soil. The Cd concentration gradients of 0 (Cd0), 5 (Cd5), 20 (Cd20), and 45 (Cd45) mg Cd kg^{-1} dry soil, which were chosen according to several previous studies (Wu et al., 2018; Dhaliwal et al., 2020), were set to understand whether *D. pinnata* can be defined as a Cd hyperaccumulator. The weighed Cd (via $CdCl_2 \bullet 2.5H_2O$) was dissolved in an appropriate amount of deionized water, and the solutions were adequately mixed with the Cd-free soils to obtain soils with targeted Cd concentrations. Actual Cd concentrations (Cd5: 6.84 mg kg^{-1}, Cd20: 20.10 mg kg^{-1}, and Cd45: 45.40 mg kg^{-1}) were measured prior to experiments. The prepared soils were equally loaded into uniform flowerpots (Li X. et al., 2022) with impermeable plastic trays to catch leachates. The soils were equilibrated for 1 month in a glass greenhouse (light: approximately 82% natural light, 12–14 h, 23–25°C; darkness: 10–12 h, 18–20°C; humidity: 40–60%) at Kunming Institute of Botany, Chinese Academy of Sciences.

Mature *D. pinnata* seeds purchased from a horticultural company (Wuhan, China) were surface-sterilized, cleaned, and sown (three seeds per pot) in the aforementioned flowerpots. One seedling was left in each pot after sprouting. Plants were watered regularly, and pots were position-changed to control for microenvironment variation. After 3 months of growth, plant roots, stems, and leaves, as well as rhizospheric soils,

were collected following published methods (Wu et al., 2018; Li X. et al., 2022). Cd^{2+} adsorbed on the root surface was removed using Na_2EDTA solution (15 mM, 20 min) (Wu et al., 2018). Three biological replicates were prepared.

2.1.2 Hydroponic experiments

To explore the molecular mechanisms of the Cd response without interference from soil, *D. pinnata* was grown hydroponically. Seeds were surface-sterilized (Li et al., 2021b) and germinated in culture dishes (25 ± 2°C, 14 h light/10 h dark). Two weeks later, seedlings in similar size were individually cultured in 50 mL plastic tubes containing 1/2 modified Hoagland nutrient solution (Zhou et al., 2016) for 15 d in the above-mentioned greenhouse. The culture medium was replaced every 3 d. Subsequently, the plants were transferred into two hydroponic boxes (l × w × h: 37.5 cm × 25.0 cm × 13.5 cm; 12 seedlings per box) with 10 L 1/2 modified Hoagland nutrient solution (renewed every 3 d). One month later, seedlings in one box were treated with 50 μM Cd^{2+} (via $CdCl_2 \cdot 2.5H_2O$), an appropriate Cd concentration that can effectively trigger changes in plant gene expression in a short time (Chen et al., 2021; Wang et al., 2022), whereas those in the other remained untreated as controls.

After 48 h of treatment, two to three top leaves and all roots of each plant were harvested separately. For transcriptomic analysis, samples were immediately frozen in liquid nitrogen and stored at −80°C. For Cd analysis, samples were cleaned for Cd^{2+} removal using Na_2EDTA solution (15 mM, 20 min) (Wu et al., 2018) and oven-dried at 80°C for 48 h. Three biological replicates were prepared for each measurement.

2.2 Measurement of Cd concentration

Cd concentrations in dried *D. pinnata* samples were measured following previously described methods (Li et al., 2017). Bioconcentration factors (BCFs), translocation factors (TFs), and Cd content in shoots and roots were calculated using published formulas (Li et al., 2021a).

2.3 Determination of soil physicochemical properties

Published methods (Li et al., 2021a; Li X. et al., 2022; Yang et al., 2022) were employed to measure the following variables in rhizospheric soils of *D. pinnata*: pH, cation exchange capacity (CEC), hydrolyzable nitrogen (HN), available phosphorus (AP), available potassium (AK), total Cd (TCd), and available Cd (ACd).

2.4 Soil bacterial community analysis

DNA extraction, 16S rDNA amplification, sequencing, and bioinformatics analyses were performed as previously described (Li et al., 2021a), with some modifications. In brief, soil microbial DNA was extracted using the HiPure Soil DNA Kit (Magen, Guangzhou, China), and the V3–V4 region of the 16S rDNA gene was amplified by PCR for Illumina NovaSeq 6000 sequencing using the primer pair 341F (5′–CCTACGGGNGGCWGCAG–3′) and 806R (5′–GGACTACHVGGGTATCTAAT–3′). The raw sequencing data were filtered to obtain clean reads, which were further merged and filtered to obtain effective tags. Thereafter, the effective tags were clustered into operational taxonomic units (OTUs). The representative sequences in each cluster were classified into organisms (confidence threshold value: 0.8) based on the SILVA database (version 132) (Pruesse et al., 2007). Alpha diversity analysis was conducted using QIIME (version 1.9.1) (Caporaso et al., 2010). Venn analysis between groups was performed in the R project VennDiagram package (version 1.6.16) (Chen and Boutros, 2011) to identify unique and common species and OTUs. Species comparison between groups was calculated by Welch's *t*-test in the R project Vegan package (version 2.5.3) (Dixon, 2003).

2.5 *De novo* transcriptomic analysis

Published methods were followed for RNA extraction and sequencing (Li et al., 2021b). Sequencing employed qualified libraries and the BGI high-throughput platform DNBSEQ-T7 (BGI, Shenzhen, China). Bioinformatics analysis of sequencing results followed previous methods (Li et al., 2021b). Briefly, the number of reads aligned to each unigene was obtained in RSEM (Li and Dewey, 2011), and the results were converted to fragments per kilobase per million bases. Next, differential expression analysis was performed in DESeq2 (Love et al., 2014). Differentially expressed genes (DEGs) were those with expression fold change > 2 and $P < 0.05$. Functional analyses (Gene Ontology [GO] terms and KEGG pathways) of DEGs were performed using the OmicShare platform (http://www.omicshare.com/tools).

Ten DEGs in *D. pinnata* roots and leaves were randomly selected for validation with quantitative real-time PCR (qRT-PCR), as previously described (Li et al., 2021b). Primer pairs and PCR product sizes are provided in Supplementary Table S1. The glyceraldehyde-3-phosphate dehydrogenase gene (Yuan et al., 2012) was used as the internal control.

2.6 Data analysis

Between-group differences were determined using one-way analysis of variance with Tukey's test ($n \geq 3$) or independent-

sample *t* test (n = 2). Principal component analysis (PCA) was generated in OmicShare (http://www.omicshare.com/tools).

3 Results

3.1 Cd tolerance and accumulation characteristics of *D. pinnata*

None of the Cd treatment (5–45 mg kg^{-1}) caused visible toxic symptoms in *D. pinnata* (Figure 1A). Additionally, biomass yields of shoots and roots remained stable after 3 months regardless of Cd concentrations (Figure 1B). Thus, *D. pinnata* appears tolerant to Cd.

Cd concentrations in *D. pinnata* tissues increased significantly ($P < 0.05$) with increasing soil Cd concentrations. Under identical soil Cd concentrations, tissue Cd concentrations were highest in leaves, followed by roots and stems (Figure 1C). The average Cd concentrations in shoots were 5.98, 26.51, and 36.35 mg kg^{-1} under Cd5, Cd20, and Cd45, respectively (Supplementary Figure S2). The average shoot Cd BCFs ranged from 0.80 to 1.32 under different soil Cd concentrations, whereas the root Cd BCFs ranged from 0.51 to 1.23 (Figure 1D). The Cd20 condition yielded the highest Cd BCF in both shoots and roots (Figure 1D). The average Cd TFs ranged from 0.81 to 1.59, increasing ($P < 0.05$) as the soil Cd concentrations increased (Figure 1E). Approximately 78.9–86.2% of the total Cd accumulated in shoots under different soil Cd concentrations (Figure 1F).

3.2 Changes in the physicochemical indices of *D. pinnata* rhizosphere soils

The rhizospheric microenvironment exhibited large differences in TCd and ACd concentrations between Cd0 and Cd45 soils (Supplementary Table S2). However, the two soils had similar pH, CEC, HN, AP, and AK concentrations (Supplementary Table S2).

3.3 Dynamics of bacterial communities in the rhizosphere of *D. pinnata*

3.3.1 Richness, diversity, and composition of bacterial communities

Raw reads from our high-throughput sequencing of the rhizospheric bacterial community were deposited in the NCBI Sequence Read Archive (SRA) (accession number: PRJNA874226). The samples generated 117,148–134,460 raw paired-end sequencing reads and 2,129–2,427 bacterial OTUs (Supplementary Table S3).

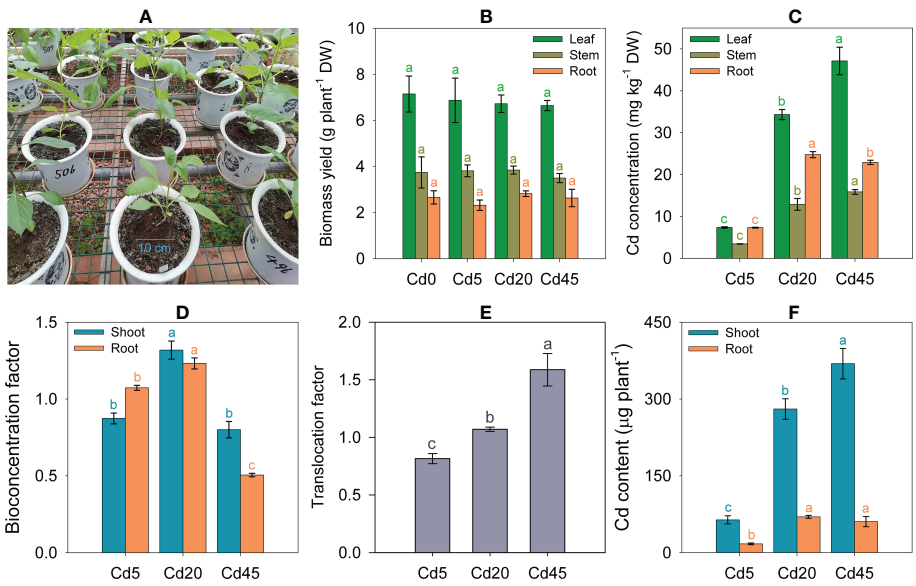

FIGURE 1
Cd tolerance and accumulation characteristics of *Dahlia pinnata*. **(A)** Greenhouse pot experiments growing *D pinnata* in soils spiked with 0 (Cd0), 5 (Cd5), 20 (Cd20), and 45 mg kg^{-1} Cd (Cd45). Biomass yields **(B)**, Cd concentrations **(C)**, bioconcentration factors **(D)**, translocation factors **(E)**, and Cd content **(F)** in *D. pinnata* under different treatments. Data represent means ± standard deviations (**B–F**: n = 3). Bars of the same color labeled with different letters indicate significant differences ($P < 0.05$, analysis of variance and Tukey's test) between groups. DW: dry weight.

The PCA showed that Cd0- and Cd45-treated samples formed distinct clusters (Figure 2A). However, bacterial communities did not differ significantly in four alpha indices (Shannon, Simpson, Chao1, and Ace) between Cd0 and Cd45 soils (Supplementary Table S4). The two soils shared 1,457 core bacterial OTUs (Figure 2B), accounting for 66.0% and 64.2% of the total OTUs in the Cd0 (2,206) and Cd45 (2,270) soils, respectively. We identified 21 bacterial phyla and 233 genera, with most of them (20 phyla and 188 genera) common across the Cd0 and Cd45 soils (Figures 2C, D). Additionally, both soils had at least one sample that contained 16 bacterial phyla (Supplementary Table S5) and 75 bacterial genera (Supplementary Table S6) with a relative abundance of > 0.1%.

3.3.2 Variations in microbial taxa between Cd0 and Cd45 soils

The Cd0 and Cd45 soils differed significantly in microbial taxa composition ($P < 0.05$, Welch's *t*-test). The abundance of the phyla Patescibacteria, Cyanobacteria, and Elusimicrobia increased in the Cd45 soil compared with that in the Cd0 soil, whereas the abundance of the phyla Bacteroidetes and Armatimonadetes decreased (Figure 2E). Additionally, 12 bacterial genera differed in abundance between Cd0 and Cd45 soils (Figure 2F).

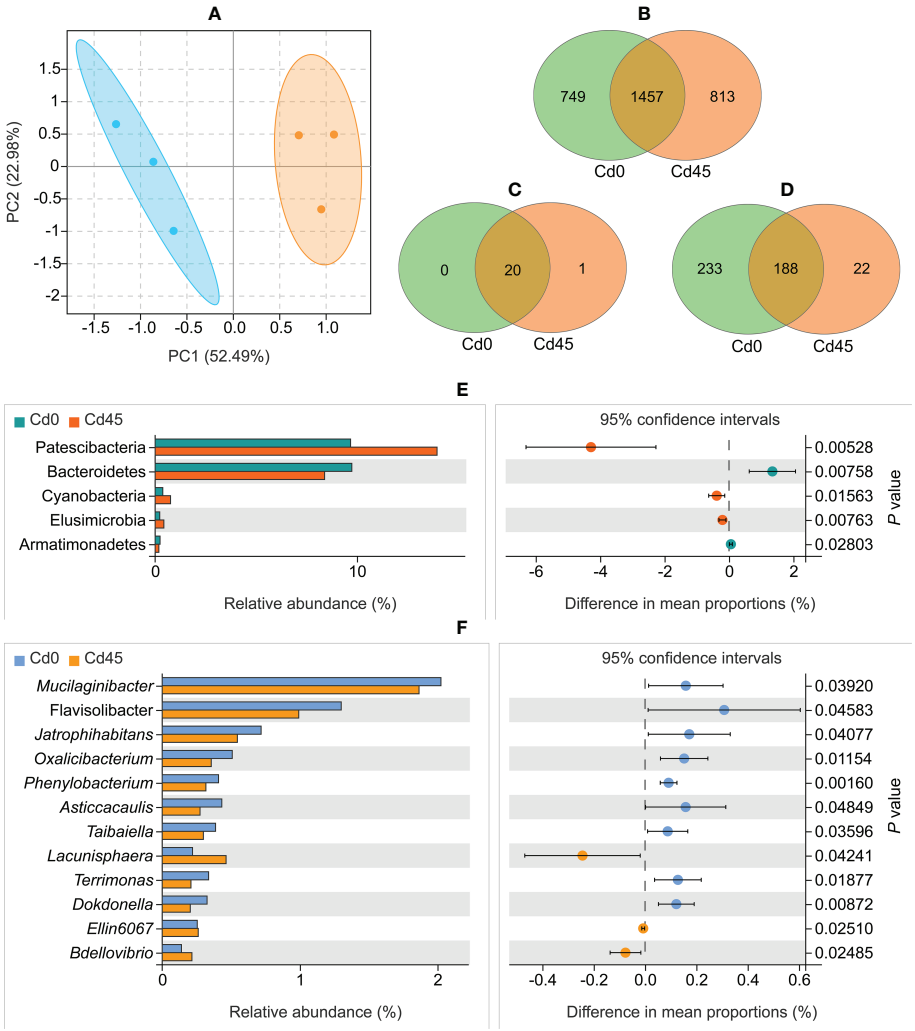

FIGURE 2
Rhizospheric bacterial community composition of *Dahlia pinnata* grown in soils spiked with 0 (Cd0) and 45 mg kg^{-1} Cd (Cd45). **(A)** Principal component analysis (PCA) of operational taxonomic units (OTUs). Venn diagram of bacterial OTUs **(B)**, phyla **(C)**, and genera **(D)** between Cd0 and Cd45 soils. Relative abundance of bacterial phyla **(E)** and genera **(F)** that differ between Cd0 and Cd45 soils. $P < 0.05$, significant using Welch's *t*-test.

3.4 Comparative transcriptomics in *D. pinnata* roots and leaves

3.4.1 Sequencing and quantitative results

After 50 mM Cd treatment for 48 h under hydroponic conditions (Figure 3A), root and leaf Cd concentrations reached 11,900 and 227 mg kg^{-1}, respectively (Supplementary Figure S3).

Approximately 67,988,424–68,000,000 raw RNA reads, which were deposited in the NCBI SRA (accession number: PRJNA811758), were generated for 12 samples, with clean reads accounting for 99% (Supplementary Table S7). High Q20 (> 97%) and Q30 (> 92%) values (Supplementary Table S7) indicated good sequencing quality. Of the 219,444 unigenes obtained, 91,692 (41.8%) were annotated in different databases (Supplementary Table S8). We quantified 40,679 (Supplementary Table S9) and 29,304 unigenes (Supplementary Table S10) in *D. pinnata* roots and leaves, respectively.

The PCA results (Figure 3B) indicated that Cd stress had a greater influence on gene expression in roots than in leaves. Under Cd stress, *D. pinnata* roots and leaves had 13,556 (4,028 upregulated and 9,528 downregulated) and 4,904 DEGs (2,260 upregulated and 2,644 downregulated), respectively (Figures 3C, D; Supplementary Tables S9 and S10). We observed tissue-specific expression for 88.8% (roots) and 68.9% (leaves) of DEGs (Figure 3E). The remaining 1,524 DEGs were identified in both roots and leaves, and 90.4% exhibited the same change patterns (Figure 3E).

3.4.2 qRT-PCR validation

Significant correlations between the RNA sequencing and qRT-PCR data in both roots (R^2 = 0.7199, P < 0.01) and leaves (R^2 = 0.6165, P < 0.01) (Figure 3F) were observed, indicating reliable RNA sequencing results in this study.

3.4.3 GO analysis

In both roots and leaves, DEGs were enriched in similar GO terms under the biological process, molecular function, and cellular component categories (Supplementary Figures S4 and S5). Nearly all enriched terms contained upregulated and downregulated genes (Supplementary Figures S4 and S5), suggesting that Cd stress exerts multiple physiological effects on *D. pinnata*.

3.4.4 KEGG pathway enrichment

The results of enrichment analysis on upregulated genes found that they were significantly enriched (P < 0.001) in 15 KEGG pathways in roots and four in leaves (Figure 4; Supplementary Table S11). The pathways were primarily associated with substance metabolism, signal transduction, and

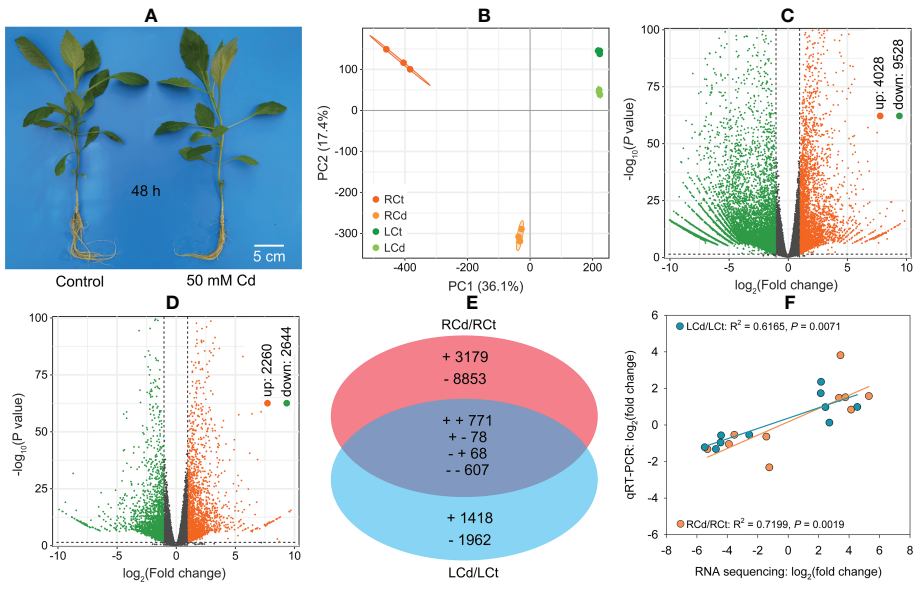

FIGURE 3
Transcriptomic analysis and qRT-PCR validation for *Dahlia pinnata* grown in control and Cd treatment (50 mM, 48 h) conditions. **(A)** Morphology of *D. pinnata* after experimental treatment under hydroponic conditions. **(B)** Principal component analysis (PCA) based on unigene abundance. **(C)** Volcano plot of differentially expressed genes (DEGs) between control and Cd-treated roots. **(D)** Volcano plot of DEGs between control and Cd-treated leaves. **(E)** Venn diagram showing DEG variation between roots and leaves. **(F)** Linear regression of gene expression changes between RNA sequencing and qRT-PCR for 10 selected genes in roots and leaves. RCt, control group of root; RCd, Cd-treated group of root; LCt, control group of leaf; LCd, Cd-treated group of leaf.

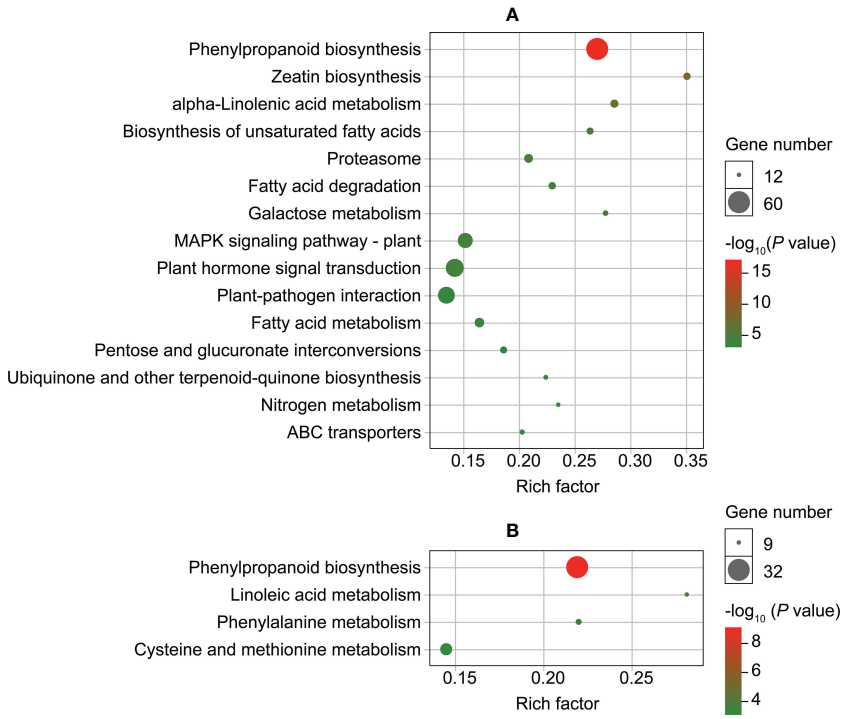

FIGURE 4
Enriched KEGG pathways ($P < 0.001$) of up-regulated genes in roots **(A)** and leaves **(B)** of *Dahlia pinnata* grown in control and Cd treatment (50 mM, 48 h) conditions.

substance transport (Figure 4). Only one pathway (phenylpropanoid biosynthesis) was enriched in both roots and leaves (Figure 4).

We selected several pathways for further analysis based on the number of enriched genes (Figure 4). For roots, the pathways were related to phenylpropanoid biosynthesis, mitogen-activated protein kinase (MAPK) signaling, plant hormone signal transduction, and plant-pathogen interaction; for leaves, pathways were involved in phenylpropanoid biosynthesis and cysteine/methionine metabolism. Many upregulated genes were involved in key reactions of these pathways (Supplementary Figures S6–S11), providing insight into the mechanisms of Cd tolerance in *D. pinnata*.

4 Discussion

4.1 Cd phytoextraction potential of *D. pinnata*

Cd tolerance and accumulation characteristics can determine plant suitability for phytoremediation (Li X. et al., 2022). Here, our experiments demonstrated that *D. pinnata* has strong Cd tolerance based on morphology and biomass accumulation in Cd-contaminated soils (Figures 1A, B). Moreover, the evidence supports *D. pinnata* as a phytoremediator because it accumulated Cd as the soil Cd concentrations rose (Figure 1C). Its appreciable Cd BCFs and TFs (Figures 1D, E) were near the thresholds of Cd hyperaccumulators (Li J. T. et al., 2018). Other promising findings are the high Cd concentrations in *D. pinnata* after short-term Cd treatment under hydroponic conditions (Supplementary Figure S3). However, the Cd accumulation capacity of *D. pinnata* seems dependent on soil Cd concentrations, given the changes in Cd BCFs and TFs under different Cd treatments (Figures 1D, E). In particular, the Cd BCF began to decline at Cd45 (Figure 1D), suggesting that Cd uptake was suppressed. The Cd transport rates in *D. pinnata* plants may be provoked by increasing Cd stress (Figure 1E), which can be interpreted as a detoxification strategy in the roots of some plants (Xue et al., 2019). Shoot Cd concentrations, BCFs, and TFs of *D. pinnata* did not reach those of Cd hyperaccumulators (Li J. T. et al., 2018). Nevertheless, our results suggest that *D. pinnata* qualifies as a Cd accumulator (Baker, 1981).

The vast majority (78.9–86.2%) of Cd accumulated in the shoots (Figure 1F), suggesting that *D. pinnata* can potentially be used for phytoextraction in a suitable range of Cd-contaminated soils. The large biomass and substantial growth rate of *D. pinnata* should be a considerable advantage compared with those of many other Cd (hyper)accumulators (Shen et al., 2021) when used for phytoextraction. Many follow-up studies

would be useful to promote the application of *D. pinnata* for Cd. For example, more efficient cultivars for phytoextraction can be screened and identified from large germplasm resources (Liu et al., 2010). Alternatively, some targeted measures can be explored to further enhance the Cd phytoextraction efficiency of *D. pinnata*.

4.2 Effects of rhizospheric microenvironments on *D. pinnata* Cd response

As the region where roots interact with soils (York et al., 2016), the rhizosphere is critical to Cd tolerance and accumulation in plants. In this study, we found that pH, CEC, and nutrients (i.e., HN, AP, and AK) were similar between the Cd0- and Cd45-treated rhizospheres of *D. pinnata* (Supplementary Table S2), suggesting a relatively stable nutrient supply for the normal growth of *D. pinnata* plants under Cd stress.

Rhizospheric microbes have a strong influence on plant responses to heavy metals in soils (Hakim et al., 2021). In this study, Cd stress did not alter the total richness or diversity of the rhizospheric bacterial community (Supplementary Table S4), which is likely attributable to the same reasons as previous studies: a dynamic equilibrium of bacterial taxa (Li et al., 2021a; Li X. et al., 2022). The stable abundance of the most dominant phylum Proteobacteria (Supplementary Table S5), which is important to soil C and N cycles (Narendrula-Kotha and Nkongolo, 2017), between the Cd0 and Cd45 soils is probably related to HN stability (Supplementary Table S2). The variation in abundance of other rhizospheric bacterial phyla (Figure 2E) illustrates a preference or sensitivity to Cd and other soil factors. Notably, the second most dominant phylum, Patescibacteria (Supplementary Table S5), has been found to increase Cd tolerance in *Photinifraseri frase* by regulating heat shock proteins (Liu et al., 2022), indicating that the upregulation of Patescibacteria (Figure 2E) may play a similar role in the Cd response of *D. pinnata*.

Some Cd accumulators recruit plant growth–promoting rhizobacteria (PGPR) to cope with Cd stress, implying PGPR abundance typically increases under Cd contamination (Wang et al., 2020; Li et al., 2021a; Li X. et al., 2022). In this study, we were unable to determine the exact functions of these bacterial genera with differential abundance between Cd0 and Cd45 soils (Figure 2F). However, we identified several important PGPR (Abhilash et al., 2016), including *Sphingomonas*, *Gemmatimonas*, *Bryobacter*, *Flavisolibacter*, *Nocardioides*, and *Bradyrhizobium*, among the top 10 abundant bacterial genera (Supplementary Table S6). All these PGPR except *Flavisolibacter* remained stable between Cd0 and Cd45 soils (Figure 2F; Supplementary Table S6). Their abundance in Cd45 soil may allow *D. pinnata* to resist Cd through multiple mechanisms (Khanna et al., 2019; Yang et al., 2022). Some of these PGPR can also regulate phytoextraction (Khanna et al., 2019; Yang et al., 2022), for instance, by affecting Cd solubility and ameliorating soil physicochemical conditions (Dary et al., 2010; Guo and Chi, 2014; Wang et al., 2019; Asaf et al., 2020). Notably, *Sphingomonas* can secrete phytochemicals as byproducts to enhance heavy metal (e.g., Cd, copper, zinc, and nickel) bioavailability (Asaf et al., 2020). Thus, the abundant *Sphingomonas* in the Cd45 soil likely contributes strongly to Cd uptake in *D. pinnata*.

4.3 Gene network in *D. pinnata* roots and leaves under Cd stress

Transcriptomics is useful for revealing the regulatory network of the Cd response in plants (Li et al., 2021b; Wang et al., 2021; Wang et al., 2022). In this study, we performed a hydroponic experiment to avoid the effects of other soil factors on our transcriptome analysis. After 48 h of Cd treatment, *D. pinnata* roots absorbed a high concentration of Cd, with a small portion transferred to the shoots (Supplementary Figure S3). This large difference in Cd concentrations is likely the cause of distinct DEGs between roots and leaves (Figure 3E). KEGG enrichment analysis was conducted using the upregulated genes to understand potential Cd tolerance mechanisms in *D. pinnata*. The results showed that various metabolic processes, signal transduction, or substance transport pathways in *D. pinnata* were significantly induced ($P < 0.001$) by Cd (Figure 4), suggesting their important roles in Cd tolerance in *D. pinnata*. In the following sections, we discuss major stress-response processes that shed light on Cd detoxification mechanisms.

4.3.1 Phenylpropanoid biosynthesis in roots and leaves

The downstream products of the phenylpropanoid metabolism pathway, such as lignins, flavonoids, and procyanidins, improve plant tolerance to heavy metals through multiple mechanisms (Ge et al., 2022). In this study, upregulated genes were highly enriched in the synthesis process of lignins in the phenylpropanoid metabolism pathway in both the roots and leaves of *D. pinnata* (Supplementary Figures S6 and S10), which may lead to an increase in the production of various lignins (e.g., syringyl lignin, 5-hydroxyguaiacyl lignin, and guaiacyl lignin). Lignins, as the main components of the secondary wall of plant cells, can fix heavy metal ions in the cell wall through their functional groups, such as carboxyl, phenolic, and aldehyde groups, to inhibit the entry of heavy metals into the cytoplasm (Li Y. et al., 2018; Su et al., 2020; Yu et al., 2023). Therefore, the results suggest that lignin-mediated cell wall compartmentalization (Figure 5) may be an important Cd detoxification mechanism in both the roots and leaves of *D. pinnata*.

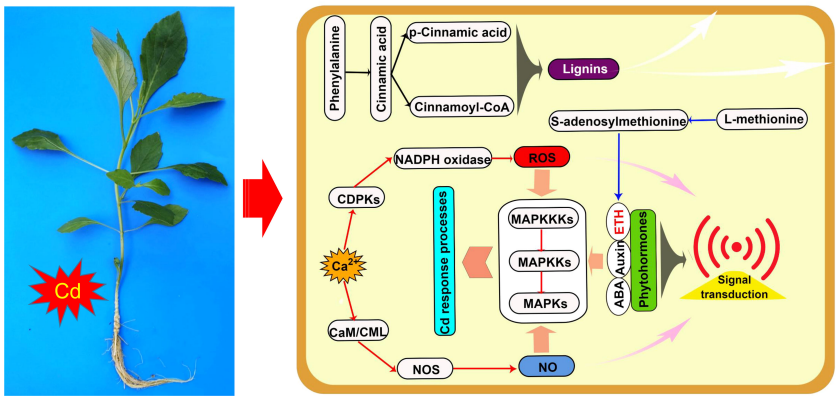

FIGURE 5
Schematic of Cd response processes of *Dahlia pinnata* grown in control and Cd treatment (50 mM, 48 h) conditions. Specific processes in roots and leaves are represented using red and blue arrows, respectively; common processes are represented using black arrows. ABA, abscisic acid; CaM/CML, calmodulin/calmodulin-like; CDPK, calcium-dependent protein kinase; ETH, ethylene; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; NO, nitric oxide; NOS, NO synthase.

4.3.2 Signal transduction networks in roots

The ability of plants to perceive, transmit, and translate stress signals into an appropriate physiological response determines their ability to tolerate heavy metals (Islam et al., 2015). Plant responses are typically modulated *via* crosstalk between signaling molecules such as Ca^{2+}, reactive oxygen species (ROS), nitric oxide (NO), phytohormones, and hydrogen sulfide (Luo et al., 2016). In this study, many upregulated genes in *D. pinnata* roots were enriched in three signaling pathways that are likely critical to Cd tolerance: MAPK signaling (Supplementary Figure S7), hormone signal transduction (Supplementary Figure S8), and plant-pathogen interaction (Supplementary Figure S9).

As a second messenger, Ca^{2+} plays an important role in intracellular signaling pathways of acclimation to heavy metals and other stressors. Ca^{2+} enters the cytoplasm through cyclic nucleotide-gated calcium channels (CNGCs) and interacts with calcium-binding proteins, including calcium-dependent protein kinase (CDPK), calmodulin (CaM), and calmodulin-binding protein kinase (Li Y. Q. et al., 2022). Of these, CDPKs are involved in phosphorylating NADPH oxidase to produce ROS (Li Y. Q. et al., 2022), whereas the Ca^{2+}/CaMs complex promotes NO synthase activity to produce NO (He et al., 2015). Here, we observed that Cd induced CNGC activity, along with Ca^{2+}-related ROS and NO production, in *D. pinnata* roots (Supplementary Figure S9). These results strengthen the hypothesis that intracellular Ca^{2+}, ROS, and NO are pivotal in plant acclimation to heavy-metal stress (Luo et al., 2016).

Phytohormones such as ethylene (ETH), abscisic acid (ABA), auxin, jasmonic acid, and salicylic acid often act as signals that trigger a plant's heavy-metal stress response (Saini et al., 2021). Unsurprisingly, Cd treatment upregulated signaling pathways in *D. pinnata* roots that involve auxin, ABA, and ETH (Supplementary Figure S9), supporting the importance of phytohormone signal transduction in the Cd stress response.

Heavy metal exposure also initiates MAPK cascades in plants as part of signal transduction (Mondal, 2022). Consistent with previous research (Li S. C. et al., 2022; Mondal, 2022), ETH, ABA, and H_2O_2 were primarily responsible for initiating MAPK cascades in *D. pinnata* roots under Cd stress (Supplementary Figure S7). Our results support the idea of crosstalk between MAPK cascades and other signaling molecules during the coordination of *D. pinnata* responses to Cd stress.

In summary, Cd stress induced a complex signal transduction network involving Ca^{2+}, ROS, NO, phytohormones, and MAPK cascades in *D. pinnata* roots (Figure 5). This network likely triggers detoxification processes that ensure *D. pinnata* tolerance to Cd.

4.3.3 Methionine metabolism in leaves

As a source of sulfur, methionine is required for the biosynthesis of glutathione and phytochelatins, which contribute to heavy-metal detoxification in plants (Thakur et al., 2022). Methionine is also involved in metal uptake and transport in plants (Mousavi et al., 2021), as well as being the immediate precursor of *S*-adenosylmethionine, itself a precursor to ETH and polyamine biosynthesis. In this study, Cd stress upregulated genes encoding enzymes key to methionine synthesis of ETH in *D. pinnata* leaves (Supplementary Figure S11). Recent evidence suggests that ETH mediates Cd resistance in plants by positively regulating flavonoid biosynthesis and antioxidant activity (Chen et al., 2022). Moreover, in the Cd hyperaccumulator *Sedum alfredii*, high ETH concentrations

postponed apoplastic-barrier formation and thus promoted Cd accumulation in root apoplasts (Liu et al., 2021). Taken together, the data strongly imply that ETH signaling based on methionine metabolism (Figure 5) is critical to regulating Cd stress responses in *D. pinnata* leaves.

5 Conclusions

This study identified *D. pinnata* as a Cd accumulator for phytoextraction based on its strong Cd tolerance capacity, as well as appreciable shoot BCFs and TFs in Cd-contaminated soils. In the *D. pinnata* rhizosphere, high Cd concentrations did not change the soil physicochemical properties but had an effect on the bacterial community composition. Notably, the increased abundance of Patescibacteria and the dominance of some Cd-tolerant PGPR likely facilitated Cd tolerance and accumulation in *D. pinnata*. Comparative transcriptomics showed that Cd significantly induced the expression of genes involved in lignin synthesis in *D. pinnata* roots and leaves. Moreover, Cd induced a sophisticated signal transduction network to initiate molecular and cellular detoxification processes in roots, whereas ethylene synthesis based on methionine metabolism likely regulates Cd responses in leaves. These results reveal the mechanism of the *D. pinnata* response to Cd stress. More research is needed to fully unlock the potential of *D. pinnata* in heavy metal phytoextraction. For example, the most suitable *D. pinnata* cultivars should be screened, and the actual Cd phytoextraction capacity in natural soils requires verification. Nevertheless, our study highlights a new Cd accumulator and provides a preliminary outline for understanding its Cd response mechanisms, with the resultant information beneficial for developing Cd phytoextraction enhancement measures.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA874226; https://www.ncbi.nlm.nih.gov/, PRJNA811758.

Author contributions

XL: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Writing - Original Draft, Visualization, Funding acquisition. BL: Investigation. TJ: Formal analysis, Writing - Review & Editing, Funding acquisition. HC: Formal analysis, Writing - Review and Editing. GZ: Formal analysis, Writing - Review and Editing. XQ: Investigation. YY: Writing - Review and Editing, Supervision. JX: Writing - Review & Editing, Supervision. All authors approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1091056/full#supplementary-material

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Muhammad Musa Khan,
Zhejiang University, China

REVIEWED BY
Zaid Khan,
Huazhong Agricultural University, China
Muhammad Amjad Bashir,
Bahauddin Zakariya University, Pakistan

*CORRESPONDENCE
Bing He
hebing@gxu.edu.cn

†These authors have contributed equally to this work and share first authorship

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Differences in the response mechanism of cadmium uptake, transfer, and accumulation of different rice varieties after foliar silicon spraying under cadmium-stressed soil

Junyang Zhao[1,2†], Baoshan Yu[1†], Xueli Wang[2], Lihong Chen[1], Kashif Akhtar[1,2], Shide Tang[1], Huaming Lu[1], Jinhua He[3], Ronghui Wen[1,2] and Bing He[1*]

[1]State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources/Guangxi Key Laboratory of Agro-Environment and Agric-Products Safety, College of Agriculture, Guangxi University, Nanning, China, [2]College of Life Science and Technology, Guangxi University, Nanning, China, [3]Soil and Fertilizer Workstation, Department of Agriculture and Rural Affairs of Nanning, Guangxi Zhuang Autonomous Region, Nanning, China

Most studies have shown that foliar silicon (Si) spraying can reduce the risk of rice quality safety caused by cadmium (Cd) contamination. However, it has recently been found that different rice varieties have different responses to Si. Therefore, we selected six rice varieties (YHSM, YXY1179, YXYLS, JLK1377, MXZ2, and YLY900) to compare the differences in the effects of leaf spray on Cd accumulation among different varieties. According to the change in Cd content in brown rice after Si application, the six rice varieties were divided into two types: Si-inhibited varieties (JLY1377, MXZ2, LY900, and YXYLS) and Si-stimulated varieties (WY1179 and YHSM). For Si-inhibited varieties, the Cd content of rice was reduced by 13.5%–65.7% after Si application. At the same time, the Cd content of the root, stem, leaf, panicle, and glume decreased to different degrees, the Cd content of the cell wall component increased by 2.2%–37.6%, the extraction state of Cd with strong mobile activity (ethanol-extracted and deionized water-extracted) was changed to the extraction state of Cd with weak mobile activity (acetic acid-extracted and hydrochloric acid-extracted), and the upward transport coefficient of different parts was reduced. For Si-stimulated varieties, Si application increased the Cd content of rice by 15.7%–24.1%. At the same time, the cell soluble component Cd content significantly increased by 68.4%–252.4% and changed the weakly mobile extraction state Cd to the strong mobile extraction state, increasing the upward transport coefficient of different sites. In conclusion, different rice varieties have different responses to Si. Foliar Si spraying inhibits the upward migration of Cd of Si-inhibited varieties, thereby reducing the Cd content of

rice, but it has the opposite effect on Si-stimulated varieties. This result reminds us that we need to consider the difference in the effect of varieties in the implementation of foliar Si spraying in remediation of Cd-contaminated paddy fields.

KEYWORDS

Cd pollution, foliar Si application, rice yield, Cd accumulation, Cd translocation

1 Introduction

Cadmium (Cd) is a heavy metal element that has a strong biological toxicity. Cd in soil mainly comes from human activities such as industrial production, agricultural irrigation, pesticide production, municipal solid waste, and transportation (Tang et al., 2018). The "Bulletin of National Survey on Soil Pollution Status" reported that the over-standard rate of agricultural land in China reached 16.1%. Heavy metal pollution accounts for about 82.8% of the total pollution, out of which Cd (7%) is higher than the other heavy metals (Ministry of Environmental Protection and Ministry of Land and Resources of the People's Republic of China, 2014). In addition, the high mobility of Cd in the soil-rice system allows it to accumulate in a large amount in the rice and transported to the grain and eventually causing the Cd rice problem. More than 60% of the population in China uses rice as staple food (Cheng et al., 2006). Therefore, the high yield, quality, and safety of rice are of very important strategic significance (Arao and Ishikawa, 2006).

Once Cd enters the soil, it is easily absorbed by crops, causing adverse effects such as slow growth and decreased yield (Nocito et al., 2011; Zeng et al., 2017). The lower concentrations of Cd can promote the growth of rice, but when the Cd content in the environment exceeds a certain threshold, it affects the absorption of water and nutrients by rice and inhibits the growth and development of rice (Rizwan et al., 2016; Chen et al., 2018; Rizwan et al., 2018). Generally manifested as growth retardation, short plants, curled leaves turning yellow and chlorosis, yellow-brown stripes, reduced tillering rate, reduced biomass, and even death, which seriously affects rice yield and quality (Rizwan et al., 2012; Hu et al., 2013; Wang et al., 2014; Shahid et al., 2017a).

The main transport processes of Cd accumulation in rice are as follows: root absorption, root-to-bud translocation caused by xylem flow, redirection transport at the nodes, and remobilization in the leaves (Uraguchi and Fujiwara, 2013). Liu et al. (2007) showed that Cd in rice was to a certain extent controlled by the absorption of Cd by plants and the transport of Cd from the root to the stem and to a greater extent influenced by the transport of Cd from the stem to the grain. Kashiwagi et al. (2009) studied the transport, accumulation, and genetic regulation of Cd in the aboveground part of rice. It was found that the content of Cd in grains was determined by the Cd accumulated in the leaves and stems before the heading stage, while from the nutrition stage to the heading stage of rice, the Cd absorbed by the roots would transfer to the upper ground and accumulate in the leaves and stems. Meanwhile, a large number of studies have shown that due to different genotypes, different rice varieties can absorb, accumulate, and distribute Cd in paddy soils between species (Ekmekci et al., 2008; Xu et al., 2010; Pan et al., 2019). However, the sensitivity and transport ability of hybrid rice to Cd were stronger than those of conventional rice, and the ability of Cd accumulation in late rice grains was stronger than that of early rice (Tang et al., 2018).

The leaf is an important nutrient organ of rice; it not only can produce organic matter (OM) through photosynthesis but also can absorb trace elements on the leaf surface (Perrier et al., 2016). Foliar control agents (FCAs) have a good effect on inhibiting the absorption of heavy metals in crops, improving the tolerance and resistance of crops to heavy metals (Vaculík et al., 2012). In recent years, it has received extensive attention because of its high bioavailability, good application effect, convenient use, and high return rate (Cheng et al., 2006). At present, the main effective components of FCAs are beneficial elements of rice such as Si, selenium, and zinc. Liao et al. (2016) showed that applying Si could significantly reduce the Cd content in brown rice through field studies. Chen et al. (2018) proved that spraying 5–25 mM nano Si could significantly reduce the Cd content in rice grains and cobs by 31.6%~64.9% and 36.1%–60.8%, respectively.

Si is the second most important element in the soil after oxygen and important in the growth and development of plants. Together with nitrogen (N), phosphorus (P), and potassium (K), it is one of the "four elements" necessary for rice. It improves the growth, development, photosynthetic capacity, resistance, and the quality of rice (Wang et al., 2010). Si can activate the antioxidant defense system, reduce oxidative damage, reduce cell membrane permeability and free radical damage to the cell membrane, improve the photosynthetic system, and remove

heavy metals in cells, thus reducing the toxicity of heavy metals (Fatemi et al., 2020; Liu et al., 2020; Tripathi et al., 2021). Li et al. (2020) found that foliar Si spraying can reduce the content of Cd in rice stems, improve the photosynthesis of rice leaves, and reduce the transport of Cd from stems to brown rice. Xu et al. (2016) used pot experiments and found that Si application reduced the content of exchangeable Cd that has a strong mobility in rice and reduced the toxic effect of Cd on rice. Guo et al. (2022) found that the concentration of Cd in various cell wall components (pectin, hemicellulose, and residues) of leaves increased by 137%~160% after Si spraying. In conclusion, the decrease of Cd content in rice by Si fertilizer application is mainly related to Si reducing the exchangeable Cd and inhibiting the transport of Cd from the root to the shoot.

Foliar spraying of Si fertilizer is a common technique to inhibit Cd uptake and accumulation in rice. In the process of large-scale implementation, we found that even in the adjacent areas with little difference in soil properties and Cd content. The application of Si fertilizer could not achieve the effect of Cd reduction in all fields of rice; on the contrary, the Cd content of rice in some fields would increase significantly. Therefore, we speculate that it may be related to rice varieties, that is, different rice varieties have different responses to Si. Therefore, we planned to compare the foliar application of Si fertilizer performance on the uptake, translocation, and accumulation of Cd and Si and yield of six different varieties of rice to further explore the response mechanism of different rice varieties to Si and its relationship with Cd accumulation. Once we master the key factors of rice response to Si, it will help to accurately guide the safe application of FCAs in Cd-polluted fields.

2 Materials and methods

2.1 Experimental site

The experiment was carried out in the glass greenhouse of Guangxi University from July 2020 to November 2020. The temperature and humidity of the greenhouse are consistent with the local natural environment with an average annual temperature of about 21.6°C.

2.2 Experiment design and treatments

Six different rice varieties were selected. All six varieties were indica rice, of which two varieties [Yuehesimiao (YHSM) and Meixiangzhan (MXZ2)] were conventional rice and the other four varieties [Yexiangyoulisi (YXYLS), Jingliangyou1377 (JLY1377), Wuyou1179 (WY1179), and Yliangyou900 (YLY900)] were hybrid rice.

The soil used for the pot experiment was obtained from the experimental field of Guangxi University in Nanning, Guangxi Zhuang Autonomous Region, China. The soil was sun dried, debris was removed, and the soil was passed through a 20-mm nylon sieve. Cadmium chloride ($CdCl_2$•$2H_2O$) is used as the source of Cd in the soil. Dissolve the Cd in water, spray it evenly on the soil, and mix it thoroughly. The soil was incubated for 1 month by repeated rehydration. The final total Cd content of the soil is 1.20 mg/kg (the pollution level is in the second category of agricultural land in China, belonging to the medium and high pollution level). The basic physical and chemical properties of soil are shown in Table 1.

The experiment was carried out in a black cylindrical plastic bucket (35 cm × 25 cm × 35 cm). Each bucket was loaded with 5.5 kg of soil. Base fertilizer (urea: 200 mg kg^{-1}; potassium dihydrogen phosphate: 130 mg kg^{-1}; potassium chloride: 200 mg kg^{-1}) was applied to the soil and thoroughly mixed. Three-leaf and one-hearted rice seedlings with similar morphology and good growth are selected and moved into pots with eight plants per pot and three replicates for each treatment. Conventional management techniques were used for top dressing and pest control. Foliar sprays were carried out twice at the tillering stage and filling stage of rice with the FCA. The main component of the FCA is nano-silica, which is provided by Foshan Tieren Environmental Protection Technology Co., Ltd. The concentration is the manufacturer's recommended dosage (1.7 g L^{-1}). Use a handheld watering to spray on the rice surface until the rice leaves are evenly moistened. The average amount of water sprayed per pot is 600 ml. The control group was sprayed with deionized water.

2.3 Sample collection and preparation

The parts of the rice samples were collected during the two growing periods (filling and maturity). The roots were soaked in 5 mmol/L EDTA-Na_2 solution for 20 min to remove the Cd on the root surface and then repeatedly rinsed with deionized water until cleaned. The aboveground parts were rinsed with tap water and then washed twice with deionized water. Some fresh leaves were frozen in liquid nitrogen and stored at -80°C to analyze the subcellular distribution and the form classification of Cd. The other samples were placed in an oven and quenched at 105°C for

TABLE 1 Basic physical and chemical property in mg/kg of soil.

pH	Organic matter	Hydrolyzable N	Available P	Available K	Total Cd	Available Cd	Available Si
6.6	18.93	182	31.72	21.36	1.2	0.51	245

30 min and then dried at 70°C to constant weight. After drying, the rice parts (roots, stems, leaves, panicles, and glumes) were separated and pulverized to determine the content of Cd and Si.

2.4 Measured index and methods

2.4.1 Determination of the basic physicochemical properties of the soil

The physical and chemical properties of the soil were determined according to the method described in the Analysis Methods of Soil Agricultural Chemistry (Bao, 2000). The pH was measured by the potentiometric method with soil/liquid ratio of 1.0:2.5. The OM content was measured by the potassium dichromate external heating method. Alkaline N was determined by the alkaline diffusion method. Available P was determined by the 0.05 mol/L HCl-0.025 mol/L ($1/2H_2SO_4$) method. Available K was leached by 1 mol/L NH_4OAC and determined by flame photometric method. Extract available Si from soil with 0.25 M citric acid and analyze it with Si molybdenum blue spectrophotometry. The total Cd content in soil was determined by 2:2:1 HNO_3:$HClO_4$:HF (v:v:v) digestion. The effective state Cd content was extracted by Diethylenetriamine Pentaacetate (DTPA) solution, and the Cd contents of the digestion solution and extract solution were determined using an atomic absorption spectrophotometer (PinAAcle 900T, PerkinElmer, USA).

2.4.2 Determination of the cadmium content in rice

Cd content determination refers to Pan Yao's method (Hu et al., 2016). The brown rice dry sample passing the 100-mesh sieve was digested with high-grade pure concentrated nitric acid by microwave digestion (microwave digestion instrument, CEM Company, MARS). The Cd content was measured with graphite furnace atomic absorption spectrophotometer (PinAAcle 900T, Platinum Elmer, USA), and the quality was controlled with reference materials.

2.4.3 Determination of the subcellular distribution of cadmium in rice leaves

Use differential centrifugation to separate leaf subcellular components (Liu et al., 2018). Weigh 0.5 g of the sample, grind it in the extract (250 mmol/L sucrose, 1 mmol/L dithioerythritol, 50 mmol/L Tris-HCl) at 4°C, collect the homogenate, and centrifuge (3,000 rpm) for 15 min; the sediment is the cell wall part, and the supernatant is the protoplast component. The supernatant (2,000 rpm) was centrifuged for 15 min to precipitate organelles. The supernatant was the soluble part containing vacuole contents, ribose, protein, etc. All operations were carried out at 4°C. Evaporate the extracted parts to dryness and determine the Cd content according to the method in *Determination of the Cadmium Content in Rice*.

2.4.4 Determination of the chemical form of cadmium in rice leaves

The chemical reagent stepwise extraction method was used to extract different forms of Cd in sequence (Shahid et al., 2017b). The five extracts are as follows: 1) ethanol extraction state: 80% ethanol; 2) water extraction state: deionized water; 3) sodium chloride extraction state: 1 mol/L sodium chloride; 4) acetic acid extraction state: 2% acetic acid; 5) hydrochloric acid extraction state: 0.6 mol/L hydrochloric acid. Weigh 0.5 g of fresh leaves, add 20 ml of extraction solution for extraction, shake for 20 h at 250 rpm in a 30°C incubator, and centrifuge for 10 min at 3,500 rpm. Collect the supernatant into the centrifuge tube, repeat the above operation twice, and merge the extracts. The sediment is extracted with the next step, and the above steps are repeated. Finally, evaporate the five extracts to nearly dry and determine the content of Cd according to the method in *Determination of the Cadmium Content in Rice*.

2.5 Statistical analysis

One-way analysis of variance (ANOVA) test was applied to study the effect of foliar Si fertilizer on the growth, yield, and accumulation of Cd and Si of different rice varieties under Cd stress. The statistical and correlation analysis of the data was performed by SPSS19.0 and Excel 2019 software. The significance analysis was performed by Duncan's test method, and figures were generated using Origin 2021.

3 Results

3.1 Effects of silicon on the rice growth and yield under cadmium stress

As shown in Table 2, under check (CK) conditions, the yields of different varieties from high to low are as follows: WY1179, JLY1377, YXYLS, YLY900, MXZ2, and YHSM. The yield of hybrid varieties is higher than that of conventional varieties. Among the four hybrid varieties, WY1179 obtained the highest yield, followed by JLY1377, YXYLS, and YLY900, and the remaining varieties reported low yield. Furthermore, there was no significant difference between the yield of rice varieties treated by Si spraying and the control, indicating that Si did not increase the yield of rice under the experimental conditions. Figure 1 also shows that compared with CK, after applying Si,

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TABLE 2 Response of foliar application of Si fertilizer on the grain yield (g pot^{-1}) of different varieties of rice.

Rice Varieties	CK treatment	Si treatment
Yuehesimiao (YHSM)	4.81 ± 1.04a	6.50 ± 1.19a
Wuyou1179 (WY1179)	12.14 ± 3.98a	13.02 ± 0.13a
Yexiangyoulisi (YXYLS)	11.26 ± 2.37a	12.47 ± 1.24a
Jingliangyou1377 (JLY1377)	11.29 ± 1.36a	12.82 ± 1.63a
Meixiangzhan2 (MXZ2)	9.30 ± 0.41a	10.58 ± 2.94a
Yliangyou900 (YLY900)	10.16 ± 1.80a	10.61 ± 2.29a

The data are shown as mean ± standard error (n = 3). Different letters indicated that there were significant differences among treatments of the same rice variety ($P < 0.05$).

the effective panicle number of YLY900 increased by 54.3% significantly, and the other five varieties had no significant change. Except for MXZ2, the plant height was significantly increased by 4.6%, and the other varieties had no significant difference. In addition, the 1,000-grain weight of the six varieties was not significantly different from that of CK ($P > 0.05$).

Compared with CK, the root dry weight of YLY900, WY1179, MXZ2, and YHSM after Si spraying increased by 34.8%~94.0%, while the root dry weight of the other varieties did not change significantly (Figure 2). In addition, compared with CK, Si application showed that the dry weight of the stems and leaves of JLY1377, YLY900, and YHSM varieties increased significantly by 69.9%–103.6%. As for the root/shoot ratio, compared with CK, WY1179 increased significantly by 123.9% after Si spraying, while JYL1377 decreased significantly by 33.7%.

3.2 Cadmium content in different parts of the rice plant

Among the six varieties under CK conditions, WY1179 and YLY900 had the highest Cd content in brown rice, and JLY1377, MXZ2, YHSM, and YXYLS varieties had the lowest Cd content. After applying Si, the Cd content in brown rice of WY1179 and YHSM increased by 15.7% and 24.1%, respectively, while that of JLY1377, MXZ2, and YLY900 decreased by 34.3%–65.7%. Therefore, the response mechanisms of different varieties to Si are different. According to the difference in the trend of Cd content in brown rice after Si application, the six varieties were divided into two categories for analysis and comparison. The first category was negatively regulated by Si to inhibit the accumulation of Cd in brown rice, called Si-inhibited varieties, such as JLY1377, MXZ2, and YLY900. Under Si application conditions, the Cd content of brown rice in YXYLS showed a decreasing trend, so YXYLS was also classified as a Si-inhibited variety. The second category is positively regulated by Si and promotes Cd accumulation in brown rice, called Si-stimulated varieties, such as WY1179 and YHSM.

Figure 3 showed that there are significant differences in Cd content in different parts of rice under the CK condition. The Cd content in the root is the highest, followed by that of the stem and panicle, and the Cd content in the leaf, glume, and brown rice is lowest. For Si-inhibited varieties, the Cd content in the roots, stems, leaves, panicles, and glumes of MXZ2 and YXYLS significantly decreased by 36.4%–79.2% after Si application, the Cd content in the stems and glumes of JLY1377 significantly

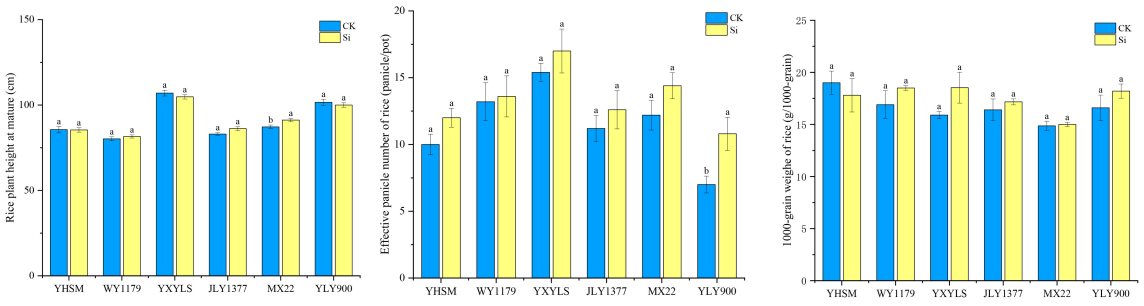

FIGURE 1
Effect of foliar spraying of silicon (Si) fertilizer on the effective number of panicle, 1,000-grain weight, and plant height of different rice varieties. The data are shown as mean ± standard error (n = 3). Different letters indicated that there were significant differences among treatments of the same rice variety ($P < 0.05$).

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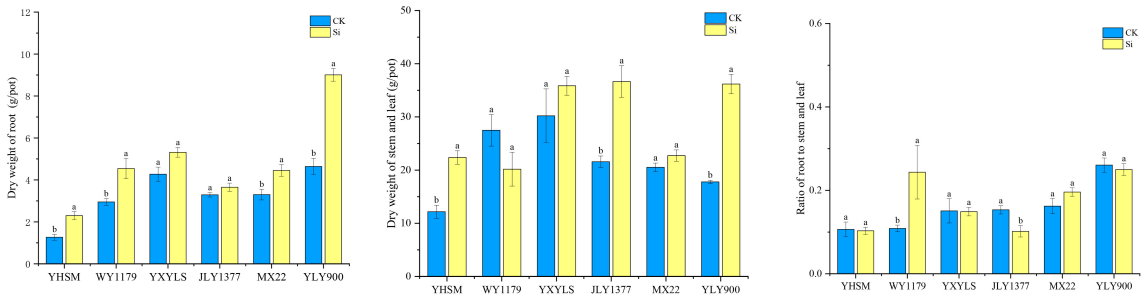

FIGURE 2
Response of foliar spraying of silicon (Si) fertilizer on the dry weight of the root, stem, and leaf and the ratio of the roots to the stems and the leaf of rice varieties at maturity stage. The data are shown as mean ± standard error (n = 3). Different letters indicated that there were significant differences among treatments of the same rice variety ($P < 0.05$).

decreased by 49.1% and 63.1%, respectively, and the Cd content in the stems and panicles of YLY900 significantly decreased by 62.2% and 42.8%, respectively. For Si-stimulated varieties, Si application significantly increased the Cd content of YHSM stems, leaves, and panicle stems by 86.2%~140.1% and the Cd content of WY1179 roots by 189.8%, with no significant change in the other parts.

The results of Cd transport coefficient analysis showed that Si spraying on leaves decreased the upward transport coefficient of Cd in Si-inhibited varieties. Among them, the decrease of the leaf-panicle transport coefficient of YXYLS and JLY1377, the stem-leaf and panicle-glume transport coefficients of MXZ2, and the root-stem and panicle-grain transport coefficients of YLY900 after foliar Si application was the main reason for inhibiting Cd accumulation in rice (Table 3). For Si-stimulated varieties, the change of mobility coefficient of each part after Si application is also different. YHSM only shows an increasing trend in the root-stem transport coefficient after Si spraying, but the stem-panicle, leaf-panicle, panicle-glume, and panicle-grain transport coefficients are decreased. Si spraying increased the panicle-glume and panicle-grain transport coefficients of WY1179 but decreased the root-stem and stem-leaf transport coefficients, indicating that YHSM mainly promoted the accumulation of Cd in brown rice by increasing the transport efficiency from the root to the shoot. WY1179 mainly promoted the accumulation of Cd in brown rice by strengthening the panicle-grain seed transport capacity.

3.3 Silicon content in different parts of the rice plant

Under CK conditions, YHSM followed by YLY900 had the highest Si content in brown rice, and WY1179, JLY1377, MXZ2, and YXYLS varieties had the lowest Si content. For the Si-inhibited varieties, compared with CK, the Si content in the roots and stems of YXYLS and MXZ2 significantly increased by 10.6%–18.9% and 28.8%–39.1%, respectively, after Si application. The Si content in the stems of YLY900 also increased significantly (Figure 4). Compared with CK, the Si content of the glume shells showed a decreasing trend, and MXZ2 decreased significantly by 13.38%. Moreover, the Si content in brown rice in YXYLS, JLY1377, and YLY900 was significantly increased by 27.7%–111.2% after applying Si. For Si-stimulated varieties, the Si content of the roots of WY1179 decreased by 19.4% significantly after Si application compared with CK. The Si content of the stems of YHSM decreased by 25.1%. The Si content of the glumes of YHSM and WY1179 decreased by 55.97% and 22.11%, respectively.

3.4 Correlation results

The correlation analysis in Table 4 shows that the Cd content in rice grains is positively correlated with that in the stems, leaves, and panicles ($P < 0.05$) but not significantly correlated with that in the glumes and roots. The Cd content in the panicles was significantly positively correlated with that in the stems ($r^2 = 0.860$) and leaves ($r^2 = 0.774$), indicating that Cd in brown rice mainly came from the panicles, and the Cd content in the panicles was positively regulated by that in the stems and leaves. The Si content in the stem was significantly negatively correlated with the Cd content in the panicles, glumes, and grains, indicating that Si in the stem inhibited Cd accumulation in the ears and above. Si inhibits the upward transport of Cd in the stems, which is of great significance to reduce the Cd content in brown rice.

3.5 Cadmium subcellular distribution in the leaf cell

It can be seen from Figure 5 that leaf Cd mainly exists in the cell wall (77.07%–93.48%), followed by soluble components (3.87%–21.47%), and only a small part of Cd

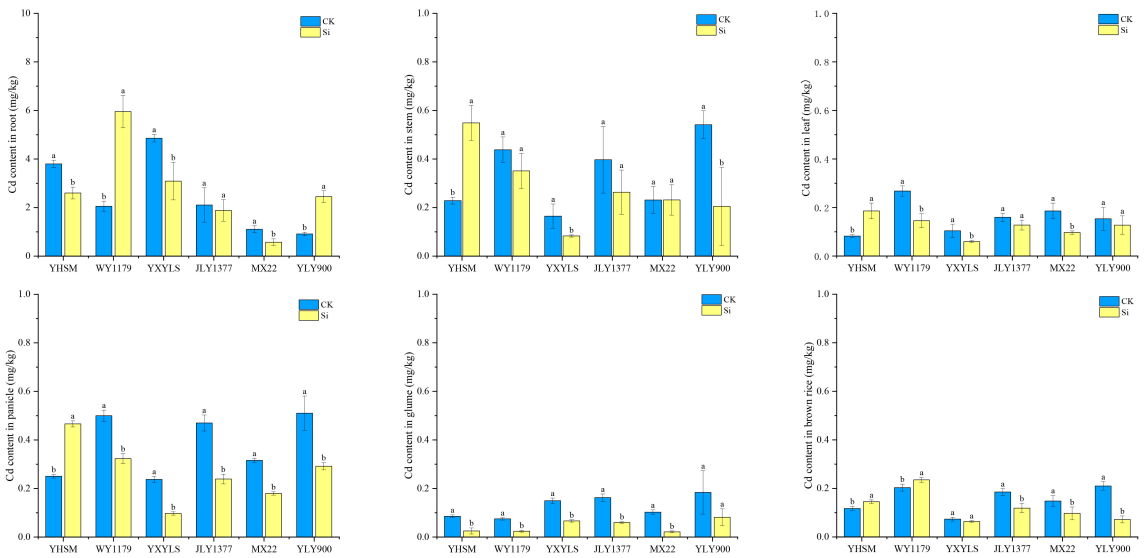

FIGURE 3
Effect of foliar spraying of silicon (Si) fertilizer on the cadmium (Cd) content in the root, stem, leaves, panicle, glumes, and brown rice of different rice varieties under Cd stress. The data are shown as mean ± standard error (n = 3). Different letters indicated that there were significant differences among treatments of the same rice variety ($P < 0.05$).

exists in the cell membrane (organs) and is the lowest (0.38%–17.09%). As for the Si-stimulating variety (WY1179), Si application decreased the distribution ratio of Cd in the cell wall and soluble components compared with CK, while Si-inhibiting varieties (MXZ2 and YLY900) showed the opposite trend by Si spraying.

3.6 Cadmium chemical form in rice leaves

The different chemical forms of Cd in the leaves of the above six rice varieties can be extracted by different extracting agents. The proportions of different Cd chemical forms are

TABLE 3 Effects of foliar application of Si fertilizer on translocation factor (TF) values for Cd in different tissues of the six rice cultivars.

Rice varieties	Treatment	$TF_{root\text{-}stem}$	$TF_{stem\text{-}leaf}$	$TF_{stem\text{-}panicle}$	$TF_{leaf\text{-}panicle}$	$TF_{panicle\text{-}glume}$	$TF_{panicle\text{-}grain}$
YHSM	CK	0.060*	0.360	1.097*	3.049	0.342*	0.467*
	Si	0.213*	0.338	0.858*	2.579	0.055 *	0.312*
WY1179	CK	0.214*	0.620	1.147*	1.878	0.151*	0.410*
	Si	0.059*	0.438	0.939*	2.315	0.077*	0.737*
YXYLS	CK	0.034	0.651	1.548	2.388*	0.633	0.444*
	Si	0.029	0.714	1.166	1.631*	0.690	0.662*
JLY1377	CK	0.188	0.446	1.298	2.942*	0.354	0.397*
	Si	0.161	0.524	0.984	1.877*	0.256	0.497*
MXZ2	CK	0.213*	0.821*	1.455	1.747	0.324*	0.473
	Si	0.411*	0.422*	0.837	2.056	0.120*	0.545
YLY900	CK	0.601*	0.280	0.932	3.385	0.338	0.428*
	Si	0.086*	1.094	2.990	2.470	0.271	0.245*

$TF_{root\text{-}stem} = Cd_{stem}/Cd_{root}$; $TF_{stem\text{-}leaf} = Cd_{leaf}/Cd_{stem}$; $TF_{stem\text{-}panicle} = Cd_{panicle}/Cd_{stem}$; $TF_{leaf\text{-}panicle} = Cd_{panicle}/Cd_{leaf}$; $TF_{panicle\text{-}glume} = Cd_{glume}/Cd_{panicle}$; $TF_{panicle\text{-}grain} = Cd_{grain}/Cd_{panicle}$. Values are means (n = 3). The * indicated that there were significant differences among treatments of the same rice variety ($P < 0.05$).

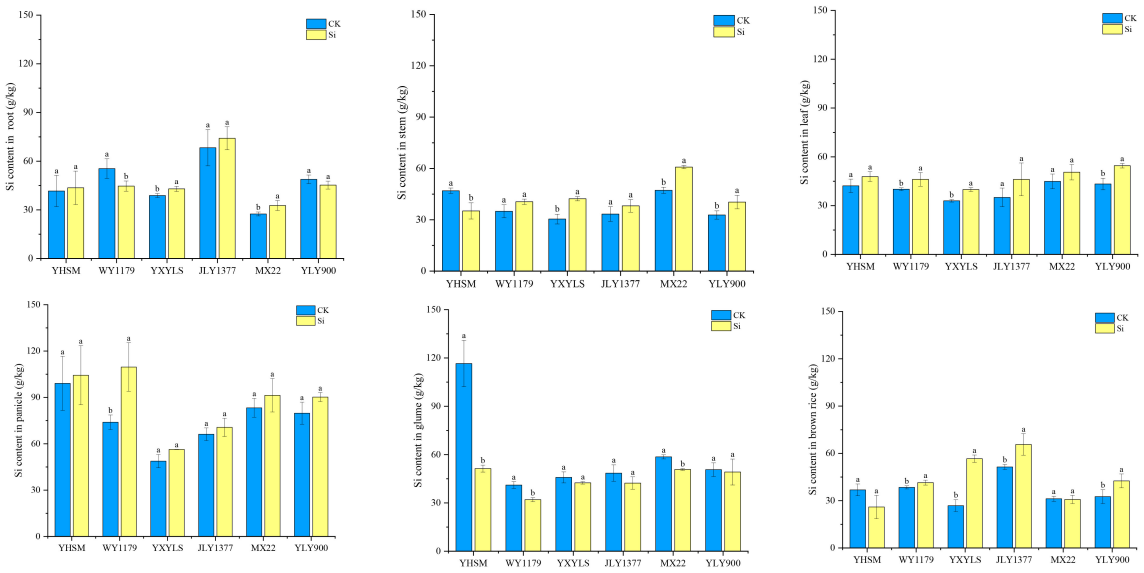

FIGURE 4
Effect of foliar spraying of silicon (Si) fertilizer on the Si content in the roots, stem, leaf, panicle, glumes, and brown rice of different rice varieties under cadmium (Cd) stress. The data are shown as mean ± standard error (n = 3). Different letters indicated that there were significant differences among treatments of the same rice variety ($P < 0.05$).

shown in Figure 6. As for Si-stimulating varieties, Cd forms with strong mobility (such as small molecular soluble salt and small molecular organic binding state) increased significantly after spraying Si, while the proportion of Cd forms with weak mobility (such as phosphate-binding state and oxalate-binding state) decreased significantly. For example, after Si spraying, the ethanol-extracted Cd of YHSM was 2.98 times higher than that of CK, while the HCl-extracted Cd was only 4.5% of CK. The proportion of various Cd forms in the leaves of Si-inhibited varieties showed opposite trends after Si spraying. For example, after spraying Si, the ethanol-extracted Cd of JLY1377 decreased by 54.77% compared with CK, and the HCl-extracted Cd increased by 473.8% compared with CK.

TABLE 4 Correlation analysis of Cd and Si content in different parts of rice varieties.

	Si_{Root}	Si_{Stem}	Si_{Leaf}	$Si_{Panicle}$	Si_{Glume}	Si_{Rice}	Cd_{Root}	Cd_{Stem}	Cd_{Leaf}	$Cd_{Panicle}$	Cd_{Glume}	Cd_{Rice}
Si_{Root}	1											
Si_{Stem}	-0.356*	1.000										
Si_{Leaf}	0.012	0.508**	1.000									
$Si_{Panicle}$	-0.109	0.392**	0.615**	1.000								
Si_{Glume}	-0.159	0.310*	0.062	0.295*	1.000							
Si_{Rice}	0.688**	-0.017	0.046	-0.169	-0.158	1.000						
Cd_{Root}	-0.032	-0.267	-0.222	0.095	0.007	0.004	1.000					
Cd_{Stem}	0.369**	-0.255	0.226	0.390**	-0.041	-0.146	-0.163	1.000				
Cd_{Leaf}	0.242	-0.217	0.146	0.213	-0.193	-0.090	-0.225	0.668**	1.000			
$Cd_{Panicle}$	0.348*	-0.446**	0.019	0.230	-0.064	-0.196	-0.180	0.860**	0.774**	1.000		
Cd_{Glume}	0.143	-0.425**	-0.392**	-0.424***	0.118	-0.002	-0.140	0.164	0.158	0.411**	1.000	
Cd_{Rice}	0.258	-0.309*	-0.092	0.266	-0.172	-0.106	0.102	0.690**	0.608**	0.737**	0.211	1.000

Correlation is the comparison between different treatments of the same variety. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

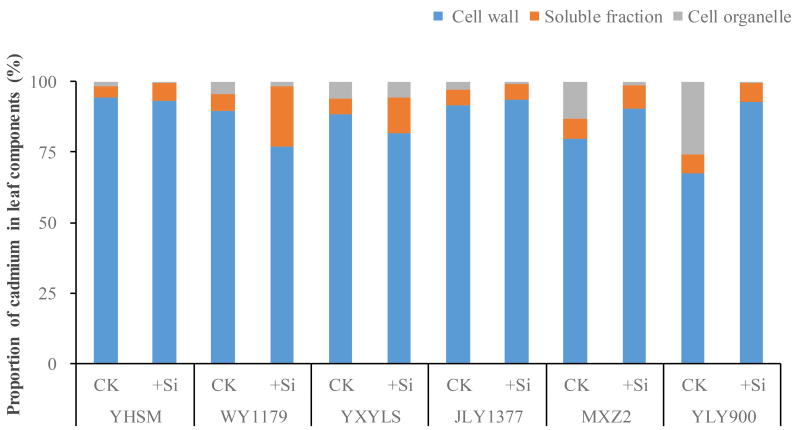

FIGURE 5
Effect of foliar spraying of silicon (Si) fertilizer on the proportion of cadmium (Cd) subcellular distributions in the leaf of different rice varieties.

4 Discussion

4.1 Effect of silicon on yielding traits of rice under cadmium stress

Cd can inhibit the growth of crops by disrupting the normal physiological and molecular mechanisms of crops (Rizwan et al., 2016), and Si spraying on the leaf surface will alleviate this restriction to some extent. In this study, foliar Si spraying promoted rice growth under Cd stress. Compared with CK, foliar Si spraying generally increased the dry weight of the roots, stems, and leaves of rice, with a higher increase in YLY900, and also increased the plant height of MXZ2 rice. Jiang and Zhang (2002) found that the increase of photosynthetic capacity and dry matter productivity of rice after the application of Si fertilizer may be the reason for the increase of dry matter weight of rice leaves, stem sheath, and panicle. Li et al. (2018) showed that exogenous Si application could reduce the inhibition of Cd stress on rice growth by promoting photosynthesis and antioxidant enzyme activity.

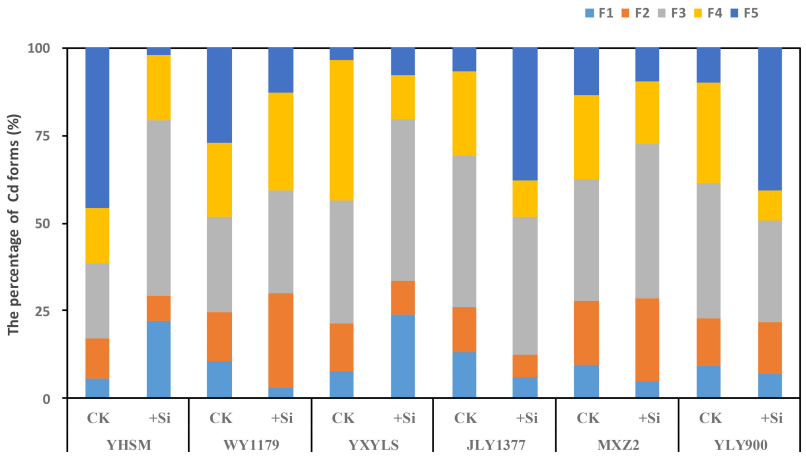

FIGURE 6
Effect of foliar spraying of Si fertilizer on the distribution proportion of cadmium (Cd) forms in the leaf of different rice varieties. F1, ethanol extraction state of Cd; F2, water extraction state of Cd; F3, sodium chloride extraction state of Cd; F4, acetic acid extraction state of Cd; and F5, hydraulic acid extraction state of Cd.

Si has a certain effect of promoting rice yield increase, but it varies with various factors (Huang et al., 2013). In this study, the results showed that the 1,000-grain weight of rice had no significant change after spraying Si fertilizer on the leaf surface, but the number of effective panicles and rice yield showed a certain increasing trend from the perspective of yield components. Wang et al. (2007) found that the effect of Si application on yield increase was mainly realized by the increase of effective panicles and grains per panicle, while 1,000-grain weight had little effect. It has also been found that Si fertilizer can improve rice yield by increasing the number of tillers, total spikelets, and seed setting grains of rice and increasing the percentage of panicles (Yang et al., 2021; Shang et al., 2009). Therefore, we speculate that the effect of spraying Si fertilizer on leaves on the number of effective panicles may be an important reason for the effect of Si fertilizer on rice yield in this experiment.

4.2 Foliar silicon spraying reduced cadmium accumulation in silicon-inhibited rice

Foliar spraying with Si slows down the toxic effects of Cd by fixing Cd in the leaves and stems. Shi et al. (2005) and Zhang et al. (2008) found that the accumulation of Si in the root can reduce the transportation of apoplast, provide metal-chelating points, and reduce the content of different forms of Cd in the apoplast, especially free Cd, thus reducing the absorption of Cd and the transfer from the root to the upper ground. It has also been pointed out that Si may affect the redistribution of Cd after it enters the rice body and inhibit the toxicity of Cd by reducing the upward transport of Cd (Wang X. M. et al., 2016). This study also found that the root-stem, stem-leaf, and leaf-panicle transport of Si-inhibited varieties was inhibited to varying degrees after Si spraying, resulting in a significant decrease in Cd content in rice panicles. Through further correlation analyses, it was found that Cd in brown rice mainly came from the ears, while Si in the stems inhibited the accumulation of Cd in the ears. Zhang et al. (2016) also found that under Cd stress, Si application significantly reduced the Cd transfer coefficient and enrichment coefficient and reduced the Cd content of brown rice. Therefore, for Si-inhibited varieties, the accumulation of Si in roots and stems increased after Si spraying, which inhibited the upward transport of Cd, thereby reducing the accumulation of Cd in rice.

The significant difference in the absorption and accumulation of Cd in different rice varieties, which is related to the subcellular and chemical form distribution of Cd, is different in rice (Li et al. 2003; Ma et al., 2015). Gu et al. (2011) believe that the highest content of Cd in the cell wall is due to the fact that the cell wall contains a large number of negatively charged groups. These groups are precipitated and complexed with positively charged heavy metal ions, so that most of the Cd is bound in the cell wall. Rice can alleviate the toxic effects of Cd by combining Cd with Si in the cell wall and changing the redox potential (Shao et al., 2017; Chen et al., 2019). Combining with the negatively charged hemicellulose form of Si can inhibit the uptake of Cd by rice cells (Zhou and Wang, 1999). The effect of Si on different migration states of Cd in rice also shows that the formation of Cd is hard to migrate and thus reduced the migration of Cd in rice (Peng et al., 2017). After foliar spraying of Si fertilizer in Si-inhibited varieties, the Cd content in the organelles decreased, and the Cd content in the cell wall increased significantly. At the same time, the ratio of the ethanol-extracted state and deionized water-extracted state with strong flow activity decreased, while the proportion of weak flow active extraction state (acetic acid-extracted state and hydrochloric acid-extracted state) increased. Therefore, for Si-inhibited cultivars, the reason for the reduction of grain Cd content after Si application may be that it reduces the Cd content in organelles, increases the adsorption of Cd on the cell wall, and promotes the refractory migration of acetic acid-extracted and hydrochloric acid-extracted Cd forms and reduced bottom-up transport of Cd in the rice, thereby reducing Cd transport to the grain and ultimately reducing Cd content in brown rice.

4.3 Foliar silicon spraying promoted cadmium accumulation in silicon-stimulated rice

Si spraying on the leaves significantly increased the Cd content in brown rice of WY1179 and YHSM. We call these rice varieties Si-stimulated. The Cd in brown rice comes from two main sources: firstly, the translocation of Cd absorbed by the roots into brown rice through xylem transport and, secondly, the reactivation of Cd accumulated in the leaves, especially the sword leaves (Tamai and Ma, 2008). Our results showed that the Si content in the stem of YHSM and the root of WY1179 decreased rather than increased after leaf spraying. At the same time, the Cd content in the stems of YHSM and the roots of WY1179 was significantly increased, and the transport coefficients of the rhizomes and panicle-grain were increased, which might promote the accumulation of Cd in rice. In addition, after Si application, the Cd content in the glume of WY1179 and YHSM decreased, indicating that Si application may reduce the distribution proportion of Cd in Si-stimulated varieties' glume, thus promoting the accumulation of Cd in rice. Our results also showed that the content of Cd in the cell wall of the Si-stimulated variety (WY1179) was significantly reduced, while the proportion of Cd in the soluble fraction was increased. In addition, the extracted Cd (acetic acid and hydrochloric acid extracts) with weak flow activity became ethanol extracts and deionized water extracts with a strong flow activity. In this study, the higher content and distribution of Cd in the ethanol-

extracted and NaCl-extracted states in the leaves of the Si-stimulated variety YHSM allowed more active Cd to be transported to the grain during the filling period, which may have contributed to its higher grain Cd content. Differences among varieties are also small in the roots, stems, and leaves but larger in the grains (Liu et al., 2007). Therefore, for Si-stimulated varieties, the increase in the Cd content of brown rice after Si application may be due to the increased Cd content in the cell organelles, reduced Cd adsorption on the cell wall and promotion of the production of the mobile active ethanol-extracted and deionized water-extracted states of hard, and increased Cd uptake by the rice root system and bottom-up transport of Cd in rice, thus facilitating Cd translocation to the grain and ultimately increasing the Cd content of brown rice.

5 Conclusions

In this study, we compared the effects of foliar spraying of Si fertilizer on the absorption, transport, and accumulation of Cd and Si as well as the yield of six different rice varieties, so as to further explore the response mechanism of different rice varieties to Si. The results showed that foliar spraying of Si fertilizer generally increased the dry weight of the root, stem, and leaf and promoted the growth of rice, but the effective panicle number and yield of rice had no significant change, which may be related to the low Si concentration. However, the response of Cd accumulation to Si in different rice varieties was significantly different. The content of Cd in the grain of Si-stimulated rice was increased by Si, while that of Si-inhibited rice was inhibited by Si. We have preliminarily clarified the mechanism of response difference. For Si-inhibited varieties, after applying Si, the content of Cd in the leaf cell wall and the chemical binding state with weak mobility are increased, the mobility and activity of Cd are reduced, and the bottom-up transport of Cd in rice straw is reduced; finally, the content of Cd in brown rice is reduced. For Si-stimulated varieties, Si application increased the content of Cd in leaf organelles and the chemical binding state with strong mobility and increased the uptake of Cd by rice roots and the transport of Cd from bottom to top in rice straw, thus promoting the transport of Cd to grains. Therefore, we suggest that future large-scale spraying of Si fertilizer on Cd-contaminated farmlands should be combined with rice varieties in order to achieve the desired effect.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

JZ and BY performed experiment and data curation, XW and LC done formal analysis and software, KA - writing review and editing, ST - Methodology, HL and JH - software, RW - writing review and editing, BH - supervision, and funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Pankaj Bhatt,
Purdue University, United States

REVIEWED BY
Benshui Shu,
Zhongkai University of Agriculture and Engineering, China
Xiangfeng Jing,
Northwest A&F University, China

*CORRESPONDENCE
Yonggen Lou
yglou@zju.edu.cn
Yaobin Lu
luybcn@163.com

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Molecular characterization and functional analysis of cytochrome P450-mediated detoxification *CYP302A1* gene involved in host plant adaptation in *Spodoptera frugieprda*

Muhammad Hafeez[1,2], Xiaowei Li[2], Limin Chen[2,3], Farman Ullah[4], Jun Huang[1], Zhijun Zhang[2], Jinming Zhang[2], Junaid Ali Siddiqui[5], Shu-xing Zhou[2], Xiao-yun Ren[2], Muhammad Imran[6], Mohammed A. Assiri[6], Yonggen Lou[1]* and Yaobin Lu[2]*

[1]State Key Laboratory of Rice Biology and Ministry of Agriculture Key Lab of Molecular Biology of Crop Pathogens and Insects, Institute of Insect Sciences, Zhejiang University, Hangzhou, China, [2]State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-products, Institute of Plant Protection and Microbiology, Zhejiang Academy of Agricultural Sciences, Hangzhou, China, [3]Integrated Plant Protection Center, Lishui Academy of Agricultural and Forestry Sciences, Lishui, China, [4]Department of Plant Biosecurity, College of Plant Protection, China Agricultural University, Beijing, China, [5]College of Agriculture, College of Tobacco Science, Guizhou University, Guiyang, China, [6]Department of Chemistry, Faculty of Science, King Khalid University, Abha, Saudi Arabia

The fall armyworm (FAW) *Spodoptera frugiperda* is a destructive and polyphagous pest of many essential food crops including maize and rice. The FAW is hard to manage, control, or eradicate, due to its polyphagous nature and voracity of feeding. Here, we report the characterization and functional analysis of the detoxification gene *CYP302A1* and how *S. frugieprda* larvae use a detoxification mechanism to adapt host plants. Results demonstrated that *CYP302A1* expression levels were much higher in midgut tissue and the older *S. frugiperda* larvae. Our current studies revealed the enhanced P450 activity in the midguts of *S. frugiperda* larvae after exposure to rice plants as compared to corn plants and an artificial diet. Furthermore, higher mortality was observed in PBO treated larvae followed by the exposure of rice plants as compared to the corn plant. The dsRNA-fed larvae showed downregulation of CYP302A1 gene in the midgut. At the same time, higher mortality, reduced larval weight and shorter developmental time was observed in the dsRNA-fed larvae followed by the exposure of rice plant as compared to the corn plant and DEPC-water treated plants as a control. These results concluded that the inducible P450 enzyme system and related genes could provide herbivores with an ecological opportunity to adapt to diverse host plants by utilizing secondary compounds present in their host plants.

KEYWORDS

P450 detoxification enzyme, spodoptera frugiperda, CYP302a1, host adaptation, RNAi

Introduction

The interaction between herbivorous pests and their host plants is frequently cited as a textbook example of co-evolution (Vandenhole et al., 2021). Feeding on plants is complicated because plants have developed a wide range of morphological and chemical defensive line tactics. To avoid or diminish feeding injury, most plants induce complex chemical defense combinations to withstand insect attack (Büchel et al., 2016; Lackus et al., 2018). These chemical compounds may serve as defensive due to their unpleasant flavors and odors, or they may be toxic or mitigate the digestibility of plant tissues (Biere et al., 2004; Rehman et al., 2012). Nonetheless, identification and response to phytochemicals (allelochemicals) released by plants to defend themselves against herbivores is an important aspect of arthropod-plant interactions (Janz, 2011; Suchan and Alvarez, 2015; Vandenhole et al., 2021). The study of the interaction between plant secondary chemistry and insect herbivores is essential in the development of a successfully integrated pest management plan.

To survive, different tactics have been evolved by adapted herbivores to deal with the defense compound present in their host plants (Heidel-Fischer and Vogel, 2015a; Rashid War et al., 2018; Yang et al., 2022). Similarly, some herbivores insects metabolize and excrete plant defense compounds they consume, but others store them in their bodies to defend themselves against all-natural enemies (Petschenka and Agrawal, 2016; Heckel, 2018). Metabolic detoxification of plant toxins is the primary strategy of herbivores occurring in three phases (solubilization), phase II (conjunction) and phase III (excretion), each with its own enzymes (Krempl et al., 2016; Stahl et al., 2018; Lu et al., 2021). For example, cytochrome P450 monooxygenases (P450s) and carboxylesterases (CarE) carry out phase I, glutathione S-transferases (GSTs) and UDP-glycosyltransferases (UGTs) phase II, and ATP-binding cassette transporters (ABC) phase III (Nauen et al., 2022; Kennedy and Tierney, 2013; Jin et al., 2019; Ullah et al., 2020). These enzymes also work on endogenous substrates like hormones and lipids to carry out additional physiological processes and housekeeping functions in insects (Ketterman et al., 2011; Feyereisen, 2012). Host plant utilization and dietary diversity have both been linked to elevating detoxification enzyme activity and mRNA expression levels of related genes (Adesanya et al., 2016; Jin et al., 2019; Israni et al., 2020). In previous research, the increased P450 and GST activity have been observed in lepidopteran larvae and plant-feeding hemipterans feeding on non-preferred or less compatible plant species (Krieger et al., 1971; Yu, 1983; Mullin, 1986; Adesanya et al., 2016; Hafeez et al., 2021a). Similarly, higher P450 enzyme activity was observed in the generalist caterpillar *Spodoptera eridania* after feeding on the carrot, a non-preferred host plant compared to the lima bean, which is a more favored host (Brattsten, 2012). In addition, a significant variation in P450, CoE and GST activities in *Bemisia tabaci* and *Popillia japonica* have been reported among different host plants that are diverse in suitability (Xie et al., 2011; Adesanya et al., 2016). More recent research suggests that a large number of P450 genes related to detoxification enzymes from insects have been isolated and characterized (Schuler, 2011; Berenbaum and Calla, 2021; Berenbaum et al., 2021; Calla, 2021). For example, gossypol-induced P450s genes *CYP9A12*, *CYP9A14*, and *CYP9A98* showed high divergence in the mRNA level of *Helicoverpa armigera* and *Spodoptera exigua* larvae (Tao et al., 2012; Hafeez et al., 2019). *CYP6B8* and *CYP321A1* in the corn earworm, *Helicoverpa* zea, can metabolize xanthotoxin, flavone, quercetin as well as a variety of other phytochemicals, indicating that this insect species has evolved systems for phytochemical detoxification (Sasabe et al., 2004; Rupasinghe et al., 2007). Additionally, *CYP6B1* and *CYP6B3* in *Pailio polyxenes*, which specializes on Rutaceae and Apiaceae, show high efficiency in metabolism of furanocoumarins in its host plants and *CYP6AS* could metabolize the flavonoid quercetin in *Apis mellifera* (Mao et al., 2009). However, it is important to investigate how selective or inducible enzyme systems could provide generalist herbivores with an ecological opportunity to utilize secondary plant compounds before expending metabolic costs for detoxification.

The fall armyworm (FAW), *Spodoptera frugiperda* is a damaging insect that feeds on a variety of essential food crops, including maize and rice (Machado et al., 2008; Gouin et al., 2017; Hafeez et al., 2021a). Since 2016, this invasive pest has spread throughout Sub-Saharan Africa, resulting in significant agricultural losses (Goergen et al., 2016; Day et al., 2017; Kenis et al., 2022) and it has also made its way into South Asia, including China, where it is also dispersing rapidly across the region (Swamy et al., 2018; Li et al., 2020). Two ecological strains of FAW have been recognized from natural populations, the so-called corn and rice strains (Pashley, 1986; Nagoshi et al., 2019). Corn strain insects are prevalent on grasses such as maize and sorghum, while, insects belonging to the rice strain appear to predominate on small grasses such as rice and Bermuda grass. Although the two strains are identical physically in the field, they do have distinct preferences for host plants and show signs of reproductive isolation (Groot et al., 2010; Dumas et al., 2015). The recent genome sequencing of this species has provided new insights into how P450s function *in vivo* and how these enzymes and related genes may be involved in the pest insect's adaptive mechanism (Gouin et al., 2017). Yet, the P450 enzymes and related genes induced by this polyphagous pest insect for host plants adaptation have not been characterized.

In this study, molecular characterization and functional analysis of cytochrome P450-mediated detoxification gene involved in host plant adaptation in *S. frugiperda* was examined after feeding on rice and corn host plants for consecutive 33 generations. Tissues and stage expression patterns of the CYP302A1 gene were also evaluated. Additionally, we investigated how cytochrome P450-specific detoxification enzyme led to larval mortality by PBO inhibitor followed by feeding on rice and corn host plants. To determine if the CYP302A1 gene functions in *S. frugiperda* host plant adaptation, a functional study of the gene was carried out using RNA interference.

Materials and methods

During August 2019, larvae of *S. frugiprda* populations were collected from two different corn fields in Ping Hu, Zhejiang Province and maintained on corn seedlings in a climate control chamber at 25 ± 2°C with a 14: 10 h light: dark photoperiod at Zhejiang Academy of Agricultural Sciences, Hangzhou, China according to (Hafeez et al., 2021a).

Reagents

7-Ethoxycoumarin, 7-hydroxycoumarin, NADPH, and Piperonyl butoxide were obtained from Sigma-Aldrich (St Louis, MO, USA). Bovine serum albumin was purchased from Thermo Scientific (Meridian Rd., Rockford, IL 61101, USA).

Insect rearing and host plant selection

Two populations were established to avoid any homogenization effect. According to our previous study, the population was reared on corn plants for 33 generations (Hafeez et al., 2021a). Larvae for control treatment were reared on an artificial diet (Poitout and Bues, 1974). Both colonies were maintained in climate chambers at 27 ± 2°C and 70%–75% relative humidity (R.H) under a 14: 10 light: dark photoperiod until adult emergence. Each population was assigned a code denoting its host plant (corn or rice).

Phylogenetic and bioinformatics analysis

We selected CYP302A1 as the representative gene to work on it based on our previous research work (Hafeez et al., 2021a). The protein sequence of *S. frugieprda* CYP302A1 was compared to other insects' publicly released protein sequences using Protein BLAST: search protein databases using a protein query (nih.gov). Based on the amino acid sequence, the protein isoelectric point (pI) and molecular mass (kDa) were calculated using ExPASy: get pI/Mw. The MEGA 7.0 software (MEGA, Tempe, AZ, USA) was used to create a phylogenetic tree based on multiple alignments of protein sequences performed by ClustalW and using the neighbour-joining algorithm with bootstrap values determined by 1000 replicates.

Tissues and stages expression analysis of CYP302A1 by RT-qPCR

The differential mRNA expression level of the CYP302A1 gene in different tissues of *S. frugiperda* larvae was analyzed after feeding on rice and corn host plants. Samples such as midguts, fat bodies were collected from larvae after feeding on rice, corn host plants and an artificial diet as a control treatment for 72 h. Similarly, thoraxes, heads and wings were collected from three days old adults respectively. A total of 30 individuals were selected from each treatment with three biological replicates (10 individuals per biological replicate). Three biological replicates were used for each experiment. Total RNA was extracted separately from all tissues (midguts, fat bodies, thoraxes, heads as well as wings from larvae and adults of *S. frugiperda)* using 1mL of TRIzolTM (Invitrogen, Carlsbad, CA, USA) and cDNA was prepared from total RNA using TransScript® OneStep gDNA Removal and cDNA Synthesis SuperMix according to the manufacturer's instructions. The primer sequences used for the candidate gene are listed in Table S1. Three biological replicates and three technical replications for each cDNA sample were used for RT-qPCR analysis. The CFX96TM Real-Time PCR Detection System (Bio-Rad Hercules, CA, USA) with the iTaq Universal SYBR Green Supermix (BIO-RAD according to the manufacturer's instructions was used for RT-qPCR analysis. The relative levels of mRNAs were quantified using three biological replicates and normalized using GAPDH (GenBank: KC262638.1) and S30 (AF400225.1) as an internal control according to the protocol described by (Bustin et al., 2009). The fold changes were determined using the $2^{-\Delta\Delta Ct}$ method followed (Livak and Schmittgen, 2001).

Measurement of P450 enzyme activity

Midguts collection and sample preparation

The midguts of the larvae were dissected at 48, 72 and 96 h after feeding on the corn, rice plant and an artificial diet (Ck) as a control treatment according to (Hafeez et al., 2021b). Larvae from each treatment were cold immobilized and their midguts were separated in 0.1 mol/L phosphate-buffered saline with pH 7.4. A total of 30 larval midguts were dissected and pooled for metabolic activity experiments for each biological replicate and stored at −80°C until enzymatic activity assays were performed.

Enzymatic activity of P450

Evaluation of P450 enzyme activity was carried out following the protocol described by (Chen et al., 2018) with some minor adjustments. According to the method described by (Chen et al., 2018), the midguts enzyme activity of *S. frugiperda* larvae was measured using 7-ethoxycoumarin (7-EC) as the substrate. The midguts of thirty *S. frugiperda* 4th-instar larvae were homogenized on ice with two millilitres of homogenization buffer 0.1 M PBS at pH 7.5. The supernatant from tubes of 2 millilitres that had been subjected to centrifugation was collected and then used for P450s activity assay. Immediately after the reaction, the concentration of 7-hydroxycoumarin in the reaction mixture was determined by employing a SPECTRA max GEMINI XS spectrofluorometer (Molecular Devices, USA) and adjusting the excitation and emission filters to 356 nm and 465 nm, respectively. At least three separate experiments were carried out for each biochemical analysis with different preparations of enzymes. The method described by (Bradford, 1976) was utilized to get the results for the protein concentration using bovine serum albumin as the standard protein. The activity was recorded as nmol p-nitroanisole/min/mg protein.

Effect of piperonyl butoxide (PBO) on larval mortality after feeding on host plants

To further confirm the possible role of metabolic detoxification enzyme in *S. frugiperda* larvae to host plant adaptation. PBO solution at the concentration of 50 mg/L was prepared in 1% (v/v) acetone. For both rice and corn groups, acetone solution containing (1 µL) of PBO was applied to the pronotum of each third instar larvae and 1% (v/v) acetone only as a control using a hand applicator. Following a post-treatment with PBO for twenty-four hours, the larvae were transferred to the host plant treatments. Each independent plant treatment (15-d-old corn and rice seedlings) had a total of 50 *S.*

frugiperda larvae with 10 replicates (5 larvae per replicate). After feeding on different rice and corn plants and control, the mortality and larval mass were recorded at 48, 72, and 96 h. Each experiment was triplicated.

Processes of preparing, quantifying, and purifying double-stranded RNA

For dsRNA synthesis, CYP302A1 with a fragment size of 365bp was amplified by PCR. The primers used for the CYP302A1 amplifications were designed to add the T7 polymerase promoter sequence to the 5 ends of each strand (Table S1). Similarly, the DEPC-treated water was used as a control treatment. The dsCYP302A1 template, which was generated through PCR and then purified, was prepared following the instructions included in the T7 RiboMax Express RNAi System Kit (Promega, Madison, WI, USA). The MEGAclearTM Kit (Ambion, Austin, TX, USA) was used to purify the resulting dsRNA. The quality of dsRNA was confirmed using 1.5% agarose gel electrophoresis, and the concentration of the final dsRNA of the target gene was measured using a NanoDrop® spectrophotometer (Thermo Fisher, Waltham, MA, USA), then the final dsRNA solution was frozen at -80 degrees Celsius until to use.

dsRNA feeding bioassays for mortality and larval growth

In this study, we used the droplet-feeding method for RNAi to prevent damage to *S. frugiperda* larvae as previously defined by (Wang et al., 2018b; Hafeez et al., 2022). The dsRNA was first diluted (250 µg/µL total volume of 500µL) in diethylpyrocarbonate (DEPC)-treated water before dsRNA feeding experiments. One-day-old 3rd-instar larvae from the Corn and Rice populations were starved for 6 h before use for feeding bioassays. Starved larvae were moved individually in sterilized 24-well tissue culture plates with 1 g of artificial diet. A total of 1µL dsRNA solution (250 µg/µL) of target CYP302A1gene was placed at the centre of each well using a 2- µL pipette. After 24 h on an artificial diet with dsRNA solution, larvae were transferred to host plant treatments. Similarly, the plants treated with DEPC-treated water were used as a control treatment. Each independent plant treatment had a total of 50 *S. frugiperda* larvae with 10 replicates (5 larvae per replicate). The larvae were transferred onto 15-d-old corn and rice seedlings. For mortality analysis, the mortality was recorded at 48, 72 and 96 h and total larval duration was assessed until pupation after feeding on different rice and corn treatments and control. Each experiment was repeated in triplicate.

RNA extraction and cDNA preparation for RT-qPCR

The differential expression and knockdown of the CYP302A1 gene were studied to validate the function of CYP302A1 in host plants adaptation. Larvae were fed on an artificial diet containing dsRNA solution for 24 h, then transferred to the host plant and control treatments for 48, 72 and 96 h. A total of 24 individual larvae were selected and 8 larvae served as a biological replicate for each treatment. Three independent biological replicates were used for each experiment as described above.

Statistical analysis

SPSS 13.0 Software Package (SPSS Inc., Chicago, IL, USA) was used to analyze all data including larval weight, larval growth, enzyme activity and transcript levels of the CYP302A1 gene. Statistically significant differences were determined by Student t-test and one-way analysis of variance followed by Tukey's HSD multiple comparison tests ($P < 0.05$).

Results

Expression Profiling of the P450 Gene at Developmental Stages and Tissues

Based on our previous research work (Hafeez et al., 2021a), we found that P450 genes shows high expression. Among all the upregulated P450 genes, we found that the CYP302A1 gene was a highly up-regulated as compared to other P450 genes as well as FPKM values showed the same expression pattern as compared to other genes among different treatments (Table S2). The mRNA expression level of the P450 gene CYP302A1 at various developmental stages and different tissues of *S. frugieprda* was measured after rearing on rice plants and corn plants for 33 generations respectively (Figure 1A).

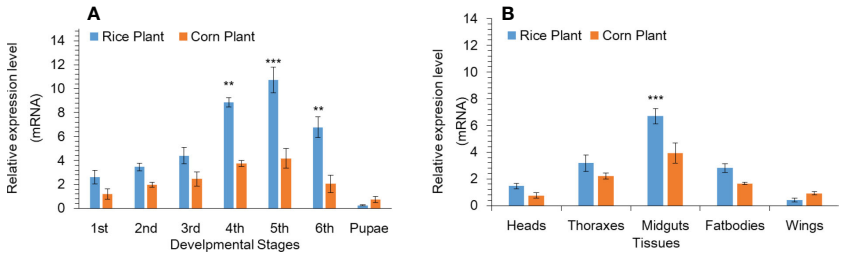

FIGURE 1
Developmental **(A)** and tissue-specific **(B)** expression pattern of *Spodoptera frugiperda CYP302A1* after feeding on rice and corn plants. Real-time quantitative RT-qPCR analysis was used to determine relative transcript levels. Data shown are mean ± SE. Treatments were compared using Student's t test. ** and *** represent P < 0.01 and P < 0.00, respectively.

Results indicated that the expression level of the P450 gene CYP302A1 was the highest in fifth-instar (rice plants; 10.7-fold) larvae among developmental stages followed by fourth-instar larvae (rice plants; 8.8 and corn plants; 3.7-fold), compared with the corn plants (5th instar; 4.2-fold and 4th instar; 3.7-fold. whereas the expression level of the CYP302A1 gene was the lowest at the first-instar (2.6-fold) and pupal stage (0.25-fold) after rearing on rice plants for 33 generations in comparison with corn plants (Figure 1A).

In addition, we analyzed the tissue distribution expression level of P450 CYP302A1 gene (Figure 1B). The midguts and fat bodies were dissected from fourth instar larvae and the different tissues were taken from 3-d old adults (heads, thoraxes and wings) after rearing on rice and corn plants for 33 generations respectively. The results showed that the mRNA expressed level of CYP302A1 was the highest in the midguts followed by fat bodies (6.7 and 2.8-fold) after rearing on rice plants compared with midguts and fat bodies (3.9 and 1.6-fold) after rearing on rice plants (Figure 1B). Whereas, the expression level of CYP302A1 was the highest in thoraxes and heads with 3.1- and 2.2-fold in the rice population compared with thoraxes and heads of the corn population respectively (Figure 1B).

P450 enzyme activity assays of 4th instar larvae

The purpose of this study was to investigate the potential role that metabolic detoxification enzyme plays in the host plant adaptation mechanism of *S. frugiperda* larvae. After 33 generations of rearing on rice and corn plants, the activity of the cytochrome P450 enzyme (P450s) in the midguts of 4th instar *S. frugiperda* larvae was evaluated. Significantly enhanced P450 activity was observed in midguts of 4th instar larvae after exposure to rice seedlings as compared to corn seedlings and artificial diet (Figure 2). After exposure to rice and corn plants, significantly increased activity of P450 enzyme was observed in the midguts by 1.56, 2.56 and 2.38 as compared to corn plants 0.92, 1.21 and 1.34 at 48, 72 and 96h respectively (Figure 2). However, the results suggest that the P450 enzyme plays a significant role in the adaptation of *S. frugiperda* to its host plant.

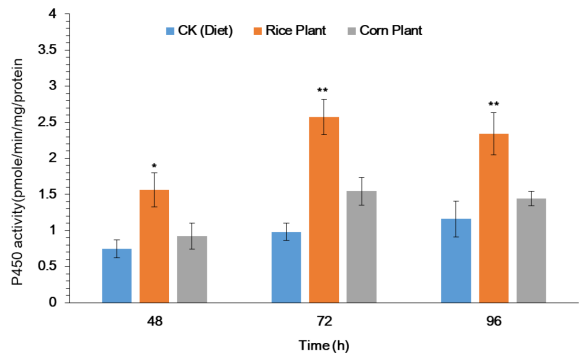

FIGURE 2
Activity of P450 enzyme in midguts of the fourth-instar larvae of FAW after feeding on rice and corn plants. The data were expressed as the means ± SE. Treatments were compared using Duncan's multiple range test. * and ** represent P < 0.05 and P < 0.01, respectively.

The effect of piperonyl butoxide (PBO) on larval mortality

To further confirm the possible role of metabolic detoxification enzyme in *S. frugiperda* larvae to host plant adaptation. PBO, a known inhibitor of the P450 enzyme was added to the diet and fed to third-instar larvae for 24 h followed by the exposure of rice and corn plants. Results indicated that the mortality of larvae pretreated with PBO was even significantly higher on rice plants as compared to the corn plant (Figure 3). Whereas, the trend of mortality was significantly higher in larvae pretreated with PBO followed by the exposure of rice plants at 72 and 96 h as compared with the larvae without treated of PBO followed by the exposure of rice plants respectively (Figure 3). This result suggesting a vital role of P450s in rice plant adaptation in *S. frugiperda*.

CYP302A1 gene characterization and phylogeny

The *CYP302A1* sequence with an open reading frame (ORF) of 1518 bp long, which encodes a protein of 495 amino acid residues. According to the translated amino acid sequence, *CYP302A1* has a theoretical pI value of 8.873 and a predicted mass of 58.84 kDa. The alignment of the deduced amino acid sequence of *S. frugiperda CYP302A1* with members of the *CYP321* family from other insect species demonstrated that it possesses the conserved motifs and conserved domains that are present in other P450 members (Supplementary Figure 1). In the comparison between *S. frugiperda CYP302A1* and the putative amino acid sequences of *Mamestra brassicae*, *Spodoptera litura*, *Spodoptera littoralis*, and *Spodoptera exigua*, the *S. frugiperda* sequence shared the highest level of similarity. (Figure 4).

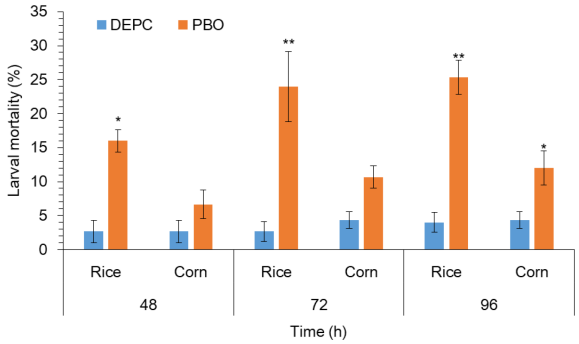

FIGURE 3
The impact of piperonyl butoxide (PBO) on larval mortality of FAW. 1 µL of PBO was applied to the pronotum of each third instar larvae and 1% (v/v) acetone only as a control using a hand applicator for 24 h followed by feeding on rice and host plants. After feeding on different rice and corn treatments and control, mortality and larval mass were recorded at 48, 72, and 96 h. Data shown are mean ± SE. Treatments were compared using Student's t test. * and ** represent P < 0.05 and P < 0.01, respectively.

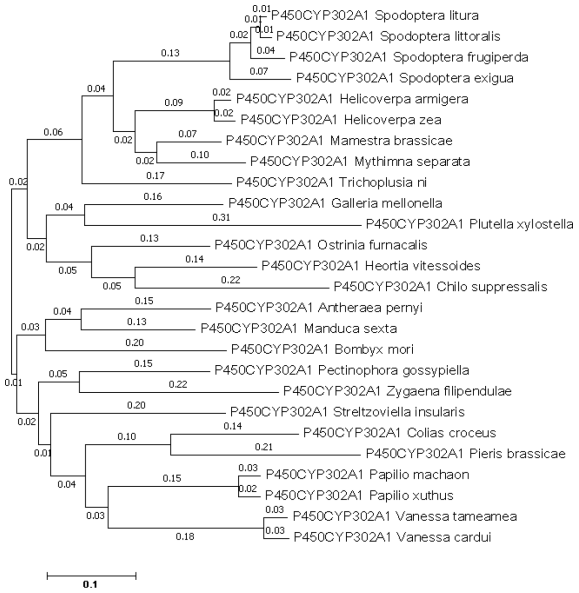

FIGURE 4
Phylogenetic analysis of *Spodoptera frugiperda* CYP302A1 and related P450s. A phylogenetic tree was constructed by the neighbor joining (NJ) method using Mega 7.0 software. The scale bar indicates 0.1 amino acid substitutions per site. Bootstrap analysis was performed with 1000 iterations.

Silencing of CYP302A1 by dsRNA

To assess whether the knockdown of detoxification *CYP302A1* gene of *S. frugiperda* plays a significant role in host plant adaptation (Figure 5). RNA-mediated down-regulation of this gene was evaluated using early third-instar larvae feeding on rice and corn plants. Pretreated larvae with dsRNA and DEPC-water treated plants as control *via* droplet feeding using an artificial diet for 24 h followed by feeding on rice and corn plants. Results showed significant down-regulation of the expression levels of the *dsCYP302A1* gene in the *S. frugiperda* larvae after feeding on rice and corn plants compared with the DEPC-water treated plants as control at 72 h (Figure 5).

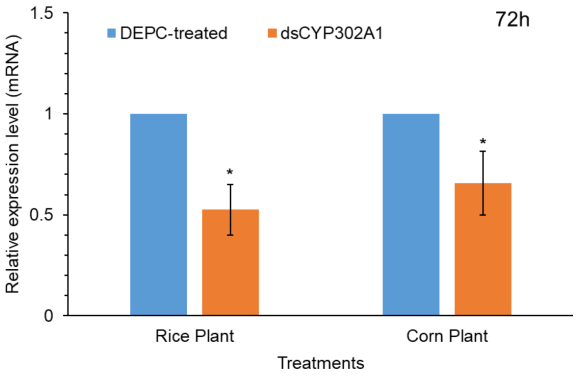

FIGURE 5
The relative mRNA transcript levels in the midguts of *S. frugiperda* larvae after feeding on dsCYP302A1 DEPC-treated water for 72 h. Data shown are mean ± SE. Treatments were compared using Student's t test. * represent P < 0.05.

Silencing effect of dsCYP302A1 on larval mortality, larval duration and weight gain

Results indicated that the down-regulation of the *dsCYP302A1* significantly increased mortality of *S. frugiperda* larvae when larvae were pretreated with dsRNA for 24 h followed by feeding on the rice plants compared with the DEPC treated water plants as a control treatment for 48 h (Figure 6A). On the other hand, no larval mortality was observed of the corn plants when larvae were pretreated with dsRNA for 24 h followed by feeding on the corn plants compared with the DEPC treated water plants as a control treatment for 48 h (Figure 6B). Similar trend was observed in larval mortality when larvae were pretreated with dsRNA for 24 h followed by feeding on the rice plants compared with the DEPC treated water plants as a

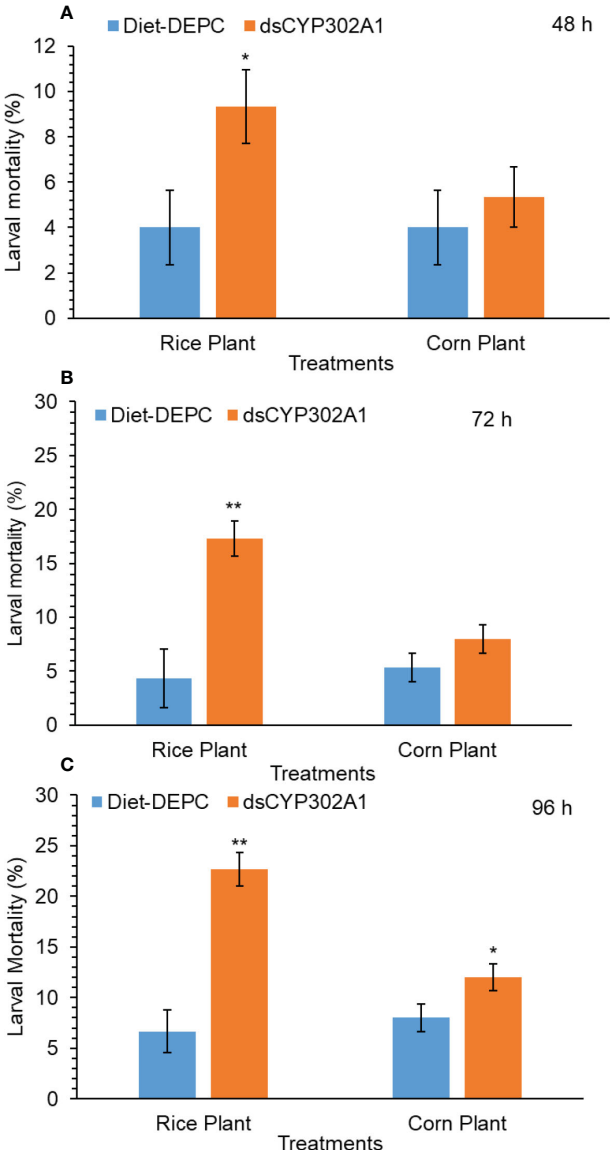

FIGURE 6
Larval mortality of S. frugiperda larvae after treated with diet containing dsCYP302A1and DEPC-treated water followed by feeding on rice and host plants for 48 **(A)**, 72 **(B)** and 96 h **(C)**. Data shown are mean ± SE. Treatments were compared using Student's t test. * and ** represent P < 0.05 and P < 0.01, respectively.

control treatment for 72 h and 96 h respectively (Figure 6C). Whereas, no significant larval mortality was found when larvae were pretreated with dsRNA for 24 h followed by feeding on the corn plants as compared with the DEPC treated water plants as a control treatment for 72 h and 96 h (Figure 6C).

The silencing effect of the *CYP302A1* gene on the *S. frugiperda* larval development and weight gain was evaluated after exposure to a dsRNA-treated diet and DEPC-treated diet as a control for 24 h followed by the feeding rice and corn plants. Our results showed that larval duration significantly increased when early third instar larvae were exposed to dsRNA-treated diets of dsCYP302A1 for 24 h followed by the feeding rice plants at 72 h (Figure 7A). While, no significant effect on larval duration was observed when early third instar larvae exposed with dsRNA-treated diets of dsCYP302A1 for 24 h followed by the feeding corn plants at 72 h (Figure 7A). Similarly, significantly decreased of the larvae weight gain was found when early third instar larvae exposed with dsRNA-treated diets of dsCYP302A1 for 24 h followed by the feeding rice plants at 72 h (Figure 7B). While no significant effect on larval weight gain was observed when early third instar larvae exposed with dsRNA-treated diets of dsCYP302A1 for 24 h followed by the feeding rice plants at 72 h (Figure 7B). Further results indicated that fewer rice plants were consumed when early third instar larvae were exposed to dsRNA-treated diets of dsCYP302A1 for 24 h followed by the feeding rice plants compared with corn plants and DEPC treated diet as control at 72 h (Supplementary Figures 2A–D).

Discussion

Herbivorous insects and their host plants use signals from each other to intensify reciprocal responses (Rashid War et al., 2018; Yactayo-Chang et al., 2020a). Upon attack by herbivorous insects, plants increase the synthesis of defensive compounds such as phytochemicals and proteins to fend them off (Zhu-Salzman and Zeng, 2015; Yactayo-Chang et al., 2020b). In response, herbivores have to evolve diverse strategies to overcome several challenges, to thrive on chemically defense compounds present in their host plant

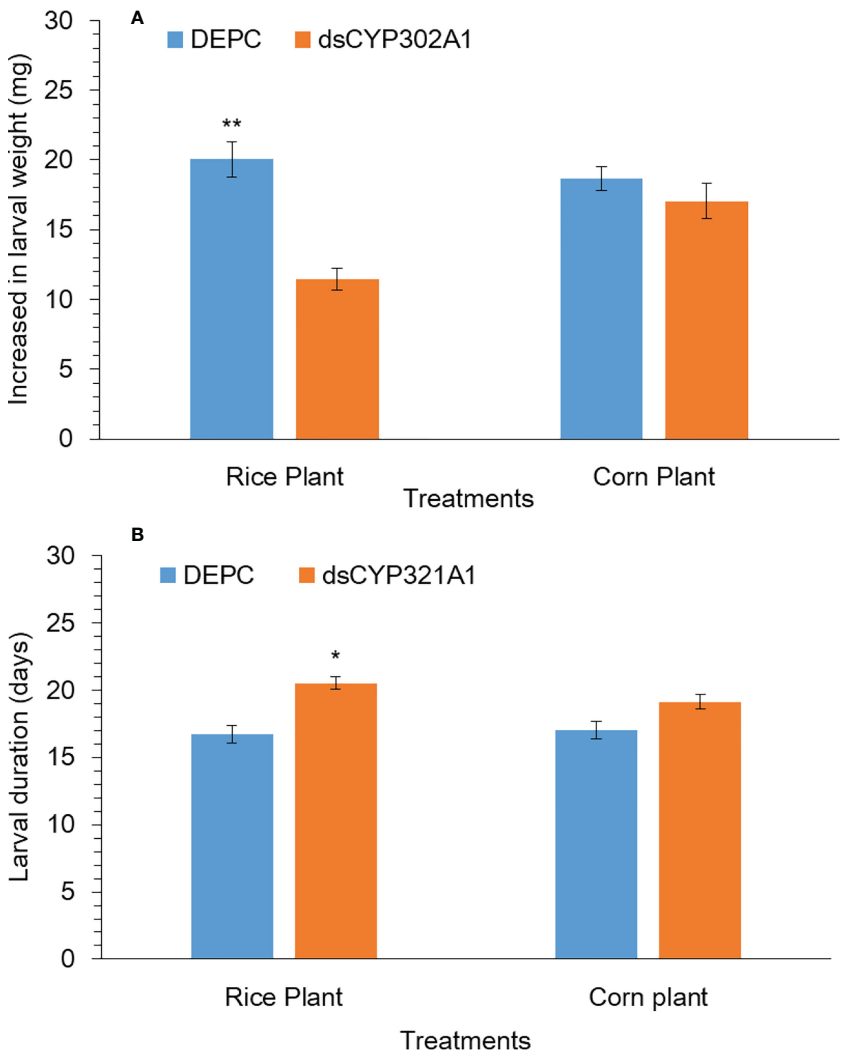

FIGURE 7
Larval weight **(A)** and larval duration **(B)** of S. frugiperda larvae after treated with diet containing dsCYP302A1and DEPC-treated water followed by feeding on rice and host plants. Data shown are mean ± SE. Treatments were compared using Student's t test. * and ** represent $P < 0.05$ and $P < 0.01$, respectively.

tissues by increasing the activity of their counteroffensive digestive and detoxification mechanism (Heidel-Fischer and Vogel, 2015b; Rashid War et al., 2018; Hafeez et al., 2021b).

In this study, we reported the characterization and functional analysis of the detoxification gene *CYP302A1* and how *S. frugieprda* larvae use a detoxification mechanism to adapt host plants. The functional and evolutionary diversification of insect P450s was likely a key factor in accelerating the unprecedented success of insects (Feyereisen, 2006b; Zhu et al., 2018). The deduced amino acid sequence of *S. frugiperda CYP302A1* aligned with other insect *CYP321* family members showed that it has conserved motifs and domains (Wang et al., 2017). In previous studies, it has been shown that P450 expression profiles vary dramatically during different developmental stages in most insects (Feyereisen, 2006b; Wang et al., 2018a). Similarly, it has been reported that the midgut fat bodies and Malpighian tubules are frequently associated with higher P450 activity (Hu et al., 2014;). In addition, the midgut is a highly crucial organ in the process of detoxification, and the genes that code for the detoxification enzymes involved in this process are frequently highly regulated in this organ (Feyereisen, 2006a; Hafeez et al., 2022). In this study, we investigated the expression profile of *CYP302A1* in different tissues and developmental stages of *S. frugiperda* by quantitative RT-qPCR. Similar to the expression patterns of *CYP6B48*, *CYP658*, and *CYP321B1* in *S. litura* larvae, which may potentially be involved in the plant allelochemicals metabolism, our findings demonstrated that *CYP302A1* expression levels were much higher in the midgut tissue and the older *S. frugiperd*a larvae. Similarly, the elevated expression level of *CYP321E*, *CYP321A8*, *CYP321A9*, and *CYP321B1* genes was reported in midguts and fat bodies of *P. xylostella* and *S. frugiperda* (Bai-Zhong et al., 2020). The enhanced expression level observed in midgut tissue and late larval instars could be attributed to a greater need for xenobiotic detoxification at this stage due to increased feeding activity (Feyereisen, 2006a; Wang et al., 2015; Israni et al., 2020). Tissue-specific expression levels of the *CYP302A1* gene in *S. frugiperda* further suggest that *CYP302A1* could be involved to adapt the host plant by detoxifying the plant xenobiotics.

Utilization of a large diversity of host plants and diet variability increase detoxifying enzyme activity in insect herbivores (Ahmad, 1983; Adesanya et al., 2016; Erb and Reymond, 2019). Elevated enzyme activities may be induced by the variety of phytochemicals present across the host pant range (Feyereisen, 2005). Our current studies revealed the enhanced P450 activity in the midguts of *S. frugiperda* larvae after exposure to rice plants as compared to corn plants and an artificial diet. Our findings are consistent with those of earlier studies; for instance, consumption of a non-preferred host plant by caterpillars of the *Spodoptera eridania* species and adult Japanese beetles induced higher P450 enzyme activities in comparison to the consumption of a more preferred host plant (Brattsten, 2012; Adesanya et al., 2016). Similarly, variation in P450 activities among five host plants that varied in suitability has been reported in *Bemisia tabaci* (B-biotype), a generalist whitefly (Xie et al., 2011). To further confirm the possible role of metabolic detoxification enzyme in *S. frugiperda* larvae to host plant adaptation, higher mortality was observed in pretreated larvae with PBO followed by the exposure of rice plants as compared to the corn plants. Our results are consistent with (Wu et al., 2021) who reported higher mortality of *H. armigera* larvae pretreated with PBO after exposure to plant volatile than control. Though, selective or inducible enzyme systems could provide generalist herbivores with an ecological opportunity to adapt to diverse host plants. This could be accomplished by generalist herbivores using secondary compounds present in their host plants before expending metabolic resources on detoxification.

Functional analysis of important genes has extensively been studied in insects using the RNAi technique (Kim et al., 2015; Choi and Vander Meer, 2019; Adeyinka et al., 2020; Hafeez et al., 2021b; Ullah et al., 2022). To further investigate if the host-plant-induced gene CYP302A1 is involved in host-plant adaptation, we fed dsCYP302A1 to *S. fugiperda* larvae to study the knockdown effects of the target gene on mortality and growth parameters. The dsRNA of target gene-fed larvae significantly showed reduced *CYP302A1* mRNA expression level in the midgut followed by the exposure of rice plant as compared to the corn plant and DEPC-water treated plants as a control. Similar to our findings, RNAi-triggered *CYPAB14*, *CYPA98*, *CYP321A7* and *CYP6B8* genes downregulation through uptake of dsRNAs have been documented for other insect species (Li et al., 2000; Mao et al., 2011; Hafeez et al., 2019; Hafeez et al., 2022). Present results indicate that the dsRNA-mediated knockdown of *CYP302A1* in the *S. frugieprda* larvae lead to higher mortality after feeding rice plant as compared to the corn plant and DEPC-water treated plants as control at 72 and 96 h. Our results provide the advocacy of previous findings by (Hafeez et al., 2019) who reported that silencing of *CYP6AB14* and *CYP9A98* genes in *S. exigua* larvae followed by feeding on 0.1% gossypol caused larval mortality. Similarly, silencing of HaAK gene in *H. armigera* using RNAi-mediated transgenic plant increased larval mortality when larvae were fed on the leaves of the transgenic plant (Liu et al., 2015). In previous reports, it has been documented that silencing of the *CYP6AE14* gene in *H. armigera* larvae and *CYP6AB14* and *CYP9A98* genes in *S. frugiperda* larvae by transgenic plant-mediated RNAi retarded larval growth and weight (2011; Mao et al., 2007; Tao et al., 2012; Hafeez et al., 2022). Similarly, the results obtained in the present work also indicated that the RNAi-mediated knockdown of the CYP302A1 gene increased larval mortality, reduced the larval weight and developmental time after exposure to a dsRNAs-supplemented diet with subsequent feeding on host plants as compared to the control.

Conclusion

In this study, we provide evidence that the insect P450 monooxygenases play a key role in host plant adaptation by detoxifying plant defense compounds. In the current study, tissue-specific expression levels of the CYP302A1 gene in *S. frugiperda* further advocate that CYP302A1 might be involved to adapt host plants. We revealed the enhanced P450 activity in the midguts of *S. frugiperda* larvae after exposure to rice plants as compared to corn plants and an artificial diet. These results concluded that the inducible enzyme system and related genes, however, could provide herbivores with an ecological opportunity to adapt diverse host plants by utilizing phytotoxins present in their host plants. We reported that the dsCYP302A1 caused mortality and had harmful effects on the growth and development of *S. frugiperda* larvae before exposure to dsRNA followed by the feeding on host pants. The harmful effects would be magnified if RNAi targeted multiple genes involved in the

P450 complex system. Further studies are needed to explore more P450 genes using RNAi-based approaches against insect pests for crop protection based on a recently developed genetic tool.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

The original study design was made by MH, XL, YGL and YL and discussed with the other authors and approved the manuscript All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1079442/full#supplementary-material

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EDITED BY
Muhammad Musa Khan,
Zhejiang University, China

REVIEWED BY
Atique ur Rehman,
Bahauddin Zakariya University, Pakistan
Osama Ali,
Menoufia University, Egypt

*CORRESPONDENCE
Peng Liu
liupeng2003@sdau.edu.cn

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The mechanism of silicon on alleviating cadmium toxicity in plants: A review

Lei Hou, Shengzhe Ji, Yao Zhang, Xiuzhe Wu, Li Zhang and Peng Liu*

College of Plant Protection, Shandong Agricultural University, Taian, Shandong, China

Cadmium is one of the most toxic heavy metal elements that seriously threaten food safety and agricultural production worldwide. Because of its high solubility, cadmium can easily enter plants, inhibiting plant growth and reducing crop yield. Therefore, finding a way to alleviate the inhibitory effects of cadmium on plant growth is critical. Silicon, the second most abundant element in the Earth's crust, has been widely reported to promote plant growth and alleviate cadmium toxicity. This review summarizes the recent progress made to elucidate how silicon mitigates cadmium toxicity in plants. We describe the role of silicon in reducing cadmium uptake and transport, improving plant mineral nutrient supply, regulating antioxidant systems and optimizing plant architecture. We also summarize in detail the regulation of plant water balance by silicon, and the role of this phenomenon in enhancing plant resistance to cadmium toxicity. An in-depth analysis of literature has been conducted to identify the current problems related to cadmium toxicity and to propose future research directions.

KEYWORDS

antioxidant, cadmium accumulation, cadmium toxicity, silicon, water balance

1 Introduction

Cadmium is a nonessential, highly toxic, heavy metal element and a widespread environmental pollutant that is persistent, nonbiodegradable and bioaccumulative even at low concentrations (Hussain et al., 2019). Upon entering the food chain, cadmium seriously threatens the health of animals and humans (Krämer and Chardonnens, 2001; Fulekar et al., 2009; Liu et al., 2019). Contamination of soil with cadmium severely affects plant growth resulting in leaf yellowing, stunted growth, reduced yield, reduced enzyme activity, reactive oxygen species (ROS) accumulation and protein denaturation (Bovetl et al., 2003; Fan et al., 2010; Gallego et al., 2012; Rizwan et al., 2017; Rizwan et al., 2018; Hamid et al., 2019). Owing to its high mobility in soil-plant systems, cadmium is easily absorbed by plants. Therefore, a reliable strategy to reduce cadmium toxicity and minimize its accumulation in plants is urgently needed to improve plant growth and ensure food safety.

After oxygen (47%), silicon is the second most abundant element (27.7%) in the Earth's crust. Although silicon is not essential for the survival of terrestrial higher plants, it plays a variety of roles to ehhance plant growth, especially under biotic and abiotic stress conditions (Epstein, 1994; Luyckx et al., 2017). Silicon can improve soil properties, such as enhancing soil microbial communities, regulating nutrient cycling, enhancing soil water retention and providing a more favorable growing environment for plants (Bhardwaj et al., 2022). Silicon is abundant in nature and often exists in inert forms that are inaccessible to plants. In contrast, soluble, bioavailable monosilicic/protosilicic acid [$Si(OH)_4/H_4SiO_4$] is typically present in soil at concentrations of (0.1–0.6 mM) (Epstein, 1994). Plants absorb silicon from the soil exclusively in the form of H_4SiO_4, which is the only plant-available form of silicon in the soil. After absorption, H_4SiO_4 is transported radially into the root cortex *via* the cytoplasm or plasmodesmata (Hodson and Sangster, 1989; Ma et al., 2006). Once H_4SiO_4 is loaded into the xylem, it is rapidly translocated to the aboveground plant parts through the transpiration stream (Lux et al., 2020). In organs such as stems and leaves, H_4SiO_4 is unloaded from the xylem into the extracellular apoplastic space. Subsequently, through transpiration and a series of biochemical reactions, H_4SiO_4 forms insoluble silica molecules and is ultimately deposited as a polymer of hydrated amorphous silica (SiO_2-nH_2O) in the cell lumen, cell wall, intercellular space or between the epidermal cell wall and the cuticle (Hodson and Sangster, 1989; Ma et al., 2006).

Silicon has been shown to benefit plant growth and reduce the toxic effects of cadmium (Ma and Yamaji, 2008; Gao et al., 2018; Hajiboland et al., 2018; Kollárová et al., 2019; Gheshlaghpour et al., 2021). In this review, recent research on the mechanism of silicon-induced mitigation of cadmium toxicity in plants is summarized. To date, studies on the mechanism of silicon-mediated mitigation of cadmium toxicity have mainly focused on reducing cadmium uptake and transport, increasing antioxidant properties, improving photosynthesis, promoting nutrient uptake and maintaining the integrity of the cell structure. This review highlights the novel hypothesis that silicon alleviates cadmium-induced damage to plants through the ability to alter water relations to dilute the cadmium concentration in plant tissues.

2 Silicon enhances plant growth under cadmium toxicity

Cadmium accumulation can severely inhibit plant growth. However, plant biomass and morphological traits, such as leaf length, leaf area, root length, root volume and root tip number, are enhanced by silicon under cadmium toxicity (Farooq et al., 2013; Rahman et al., 2021c; Seyed, 2022) (Table 1). Disruption of photosynthesis is a major impact of cadmium toxicity, which can be alleviated by silicon, for example, in rice (Nwugo and Huerta, 2008; Li et al., 2018), cucumber (Feng et al., 2010), cotton (Farooq et al., 2013), maize (Vaculík et al., 2015), wheat (Sun et al., 2016; Shi et al., 2018) and lupin (Sun et al., 2016). Chloroplasts are organelles that perform photosynthesis, and their ultrastructure plays a vital role in this process. With the accumulation of cadmium in tissues, the concentration of ROS increases, and the cell membrane system is

TABLE 1 Effect of silicon on plant growth and development to plants under cadmium poisoning.

Species	Silicon concentration	Cadmium concentration	Effect	Reference
Pea	100, 200, 300 ppm Si	50, 100 mg kg^{-1} $CdCl_2$	The plant height↑by 37.45% and 36.26%, leaf area↑by 33.99% and 31.87%, dry weight of shoot↑by 31.48% and 37.16, and root dry weight↑by 36.49% and 29.97%, at 300 ppm exogenous Si. The levels of chlorophylls (a and b)↑.	(El-Okkiah et al., 2022)
Wheat	25, 50, 100 mg kg^{-1} SiNPs	7.67 mg kg^{-1} Cd	The plant height↑by 5.0%, 17.6%, 25.2%, at 25, 50, 100 mg kg^{-1} Si. Chlorophyll a content↑by 17.2%, 28.5%, 44.3%, at 25, 50, 100 mg kg^{-1} Si. Chlorophyll b contents↑by 21.6%, 43.5%, 65.6%, at 25, 50, 100 mg kg^{-1} Si.	(Khan et al., 2021)
Rice	1, 2, 4, 6 g kg^{-1} $Na_2SiO_3{\cdot}9H_2O$	0.53, 3.51 mg kg^{-1} Cd	Addition of 4 g kg^{-1} Si increased the net photosynthetic rate (27%; 45%) of cadmium-poisoned (0.53; 3.51 mg kg^{-1}) plants.	(Cipriano et al., 2021)
Pepper	2 mM Na_2SiO_3	0.1 mM $CdCl_2$	The shoot, root and total plant dry mass↑by 55.3%, 31.6% and 50.3%, at 2.0 mM Si. The chlorophyll a and b contents and efficiency of photosystem II content↑by 32.6%, 23.0% and 26.4%, at 2.0 mM Si.	(Kaya et al., 2020)
Rice	1.5 mM $NaSiO_3{\cdot}9H_2O$	100 µM $CdCl_2$	The aboveground biomass↑, underground biomass↑, shoot and root lengths↑, the chlorophyll content↑by 9.7%.	(Chen et al., 2019)
Wheat	300, 600, 900, 1200 mg L^{-1} SiNPs	0.93 mg kg^{-1} Cd	The shoot length and grain weight↑by 14, 30, 43, 51%, at 300, 600, 900, and 1200 mg L^{-1} Si. The grain weight↑by 24%, 43%, 52%, 62%, at 300, 600, 900, and 1200 mg L^{-1} Si. The shoot dry weight, root dry weight, shoot length, grain weight, spike length, spike dry weight↑by 66, 67%, 51%, 62%, 53%, 66%, at 1200 mg L^{-1} Si. The photosynthetic rate↑by 79%, at 1200 mg L^{-1} Si.	(Hussain et al., 2019)
Rice	0.03% w/w K_2SiO_3	0.52 mg kg^{-1} Cd	The shoot and root dry weights↑. The photosynthetic rate↑by 83.9% with spliting application of Si at three growth stages (S1+S2+S3).	(Rehman et al., 2019)

(Continued)

TABLE 1 Continued

Species	Silicon concentration	Cadmium concentration	Effect	Reference
Wheat	3 mM $K_2SiO_3{\cdot}nH_2O$	2 mM Cd^{2+}	The Si applied as soil addition was the best treatment. The shoot length↑by 69.8%, leaf area↑by 82.7%, seedling FW↑by 86.8% and seedling DW↑by 107.7%. The net photosynthetic rate↑by 62.5%.	(Howladar et al., 2018)
Wheat	1 mM $Na_2SiO_3{\cdot}9H_2O$	5, 20 μM $CdCl_2{\cdot}3/2H_2O$	Addition of Si increased the above-ground dry weight (47%) of cadmium-poisoned (5 μM) plants. Addition of Si increased the net photosynthetic rate (27%; 45%) of cadmium-poisoned (5; 20 μM) plants.	(Shi et al., 2018)
Rice	120 mg L^{-1} Na_2SiO_3	1, 5 mg L^{-1} Cd	Addition of Si increased the above-ground dry weight (51%;81%) of cadmium-poisoned (1; 5 mg L^{-1}) plants. Applied Si can restore the morphology and structure of chloroplasts.	(Guo et al., 2018)
Wheat	0.6 mM $SiO_2{\cdot}nH_2O$	15 μM $CdCl_2{\cdot}H_2O$	Shoot dry matter of Sehar-2006↑by 16%. The photosynthetic rate of Sehar-2006↑by 44%.	(Naeem et al., 2018)
Rice	42 mg kg^{-1} $K_2SiO_3{\cdot}nH_2O$	50 mg kg^{-1} $CdCl_2{\cdot}2.5H_2O$	Shoot dry weight↑by 28% in Feng-Hua-Zhan. Shoot dry weight↑by 48% in Hua-Hang-Si-Miao. The total chlorophyll content↑by 75%. The chlorophyll fluorescence parameters by↑73%.	(Huang et al., 2018)
Tobacco	1, 4 g kg^{-1} $Na_2SiO_3{\cdot}H_2O$	1, 5 mg kg^{-1} $CdCl_2{\cdot}H_2O$	Root dry weight↑by 17% (Cd1+Si1 compared to Cd1). Stem dry weight↑by 44% (Cd5+Si1 compared to Cd5). Leaf dry weight↑by 70% (Cd5+Si4 compared to Cd5). The total biomass↑by 47% and 49% (Cd5+Si1 and Cd5+Si4, compared to Cd5). The contents of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids↑by 40.4%, 23.9%, 35.2%, and 41.1% (Cd5+Si4 to Cd5).	(Lu et al., 2018)
Gladiolus	200 mg L^{-1} Si	50 mg kg^{-1} $CdSO_4{\cdot}8H_2O$	The above-ground and root dry weight↑by 22% and 11%. The photosynthesis↑.	(Zaheer et al., 2018)
Maize	5 mM Na_2SiO_3	5, 50 μM Cd $(NO_3)_2{\cdot}4H_2O$	Addition of Si increased above-ground dry weight (32%; 21%) of cadmium-poisoned (5; 50 μM) plants. The addition of Si increased the net photosynthetic rate of Cd-poisoned plants.	(Vaculík et al., 2015)
Cotton	1 mM Na_2SiO_3	1, 5 μM $CdCl_2$	Addition of 1 mM Si increased root dry weight (25%; 62%), stem dry weight (26%; 35%), and leaf dry weight (31%; 57%) of cadmium-poisoned (1; 5 μM) plants. The addition of 1 mM Si increased the net photosynthetic rate (90%) of cadmium-poisoned (5 μM) plants.	(Farooq et al., 2013)

"↑"="increased".

damaged, especially when the chloroplast membrane is damaged, which will inhibit photosynthesis (Feng et al., 2010; Pereira AS et al., 2018). Silicon application has been reported to alleviate cadmium-induced cell ultrastructure damage in a variety of plant species (Zhao et al., 2022).

In rice, silicon ameliorates cadmium-induced changes in chloroplast ultrastructure by ensuring the integrity of chloroplasts and membranes (Guo et al., 2018). In addition, silicon-enhanced regeneration of cell walls by maize protoplasts may also be used to maintain chloroplast structure (Kollárová et al., 2019). Cadmium negatively affects the formation of cystoids in the chloroplasts of bundle sheath cells, which is mitigated by silicon and the improved cystoid formation may contribute to enhance photosynthesis and then increase biomass (Vaculík et al., 2015). Cadmium inhibits the activity of chlorophyll synthase and increases the activity of chlorophyllase, thus decreasing chlorophyll content (Ekmekçi et al., 2008; Vaculík et al., 2015). Silicon increases the content of photosynthetic pigments in tobacco under cadmium toxicity and improves the efficiency of light energy utilization (Feng et al., 2010; Luyckx et al., 2021). Cadmium toxicity also causes changes in leaf structure, stomatal size and density, which ultimately inhibit photosynthesis (Shi and Cai, 2008). However, in the presence of silicon, stomatal density is increased and results in a high gas diffusion rate (Cipriano et al., 2021). Moreover, silicon deposition is beneficial for the plant to maintain an erect habit, especially under stress and to promote leaf positioning favorable for light interception and efficient photosynthesis (Epstein, 1994).

3 Silicon reduces the uptake of cadmium transport by plants

The phytotoxic effects of cadmium present in soil are not exerted until it is absorbed by plants and transported to various tissues and organs. There are two means for cadmium to enter the root: the apoplast pathway and the symplast pathway (Redjala et al., 2009; Song et al., 2017). In the apoplast pathway, cadmium present in the soil is absorbed by plants roots *via* free diffusion; however, instead of entering the cells, cadmium passes through voids in the cell walls of rhizodermis and cortex to enter the xylem and phloem (Degryse et al., 2006; Tao et al., 2019). The symplast pathway refers to the transport of cadmium through plasmodesmata from one cell (protoplast) to the other using cytoplasmic continuum; and cadmium transport across membranes, this process is mediated by transporters located in the cell wall (Kreszies et al., 2018). Silicon interferes with these two transport pathways in various ways, inhibiting the accumulation of cadmium in plants, thereby

reducing its harmfulness to many plants (Kabir et al., 2016; Song et al., 2021).

3.1 Formation of physical barriers (inhibition of the apoplast pathway)

The formation of a physical barrier in the apoplast space reduce cadmium transport rates, thereby reducing the accumulation and distribution of cadmium in the cytoplasm (Ye et al., 2012; Zhang et al., 2014; Luyckx et al., 2017; Riaz et al., 2022). Silicon creates a physical barrier through the formation of specific cells (siliceous cells) that reduce the uptake and transport of cadmium in plants (Ma and Yamaji, 2008). Siliccon is deposited in root tissues through three main modes: (1) impregnation of the endodermal cell wall; (2) formation of silica aggregates associated with the inner tangential wall of the endodermal cells;and (3) formation of silica aggregates or phytoliths in specific cells associated with thick-walled sclerenchymatous tissues (Lux et al., 2020). After its absorption by plant roots exclusively as H_4SiO_4, silicon binds to cell wall components including hemicellulose, pectin, callose, cellulose and dextran to form SiO_2 precipitates (Guerriero et al., 2016). Experiments on rice suspension cells show that hemicellulosic polysaccharides in the cell wall exhibit greater silicon-binding capacity than pectic polysaccharides (Ma et al., 2015). Silicon significantly increases the content of total polysaccharides and their components (pectin and cellulose) in the root cell wall and improves the ability of cadmium to bind to pectin and cellulose, which decreases cadmium transport efficiency through the apoplast pathway (Cai et al., 2022). The specific amino acid composition of cell-wall-localized proteins may also lead to silicification (Guerriero et al., 2016). Silicon increases the cation exchange capacity of the cell wall; cadmium binds to the cell wall to a greater extent in the presence of silicon than in its absence (Lukačová et al., 2013). Heavy deposition of silicon near the cortex, as aggregates or phytoliths, may reduce the cell wall porosity of the internal tissues of the root, especially in the inner cortex (Lux et al., 2020). Massive deposition of SiO_2 near the endodermis physically blocks plasmodesmata flow through the root, thereby inhibiting the plasmodesmata transport of cadmium to reduce its toxic effects on plant cells (Shi et al., 2005).

In addition, the chemically heterogeneous walls resulting from silicon modification may provide additional binding sites for cadmium (Liu et al., 2013a). In rice and maize, silicon application enhances cell wall suberization and lignification and promotes Casparian strip formation (Fleck et al., 2011; Vaculík et al., 2012). In wheat plants exposed to long-term cadmium stress, silicon promoted suberin deposition in the root endodermis and decreased cadmium concentration in the apoplastic fluid of the shoot by almost 38% (Wu et al., 2019). Additionally, the ratio of root endothelium cell wall to the whole cell volume was higher in the cadmium+silicon treatment than in the cadmium-only treatment (Lukačová et al., 2013).

More than 90% of the H_4SiO_4 taken up by roots is transported to the aboveground organs, along with the transpiration water flow, through the xylem. In this process, silicon is also deposited in the leaf epidermal cell wall, which decreases the efficiency of cadmium transport (Lux et al., 2003; Keller et al., 2015). When cadmium reaches the leaf, silica aggregates in the cell wall, as a physical barrier, suppressing cadmium from entering the cells (Farnezi et al., 2020).

3.2 Downregulation of cadmium uptake and transport-related genes (inhibition of the symplast pathway)

In addition to the formation of physical barriers in the cell wall, silicon also affects the expression of genes involved in cadmium uptake and transport. The main cadmium transporter genes encode proteins belonging to the ZRT-like protein (ZIP), IRT-like protein (IRT), natural resistance-associated macrophage protein (Nramp), yellow stripe-like transporter (YSL), low affinity cation transporter (LCT), P-type adenosine triphosphatase (ATPase, HMA) and cation diffusion facilitator (CDF) families. Among these proteins, ZIPs, IRTs, Nramps, YSLs and LCTs mediate cadmium uptake from the soil by roots and its transport to aboveground plant parts, while HMAs and CDFs are involved in the efflux of cadmium to the cytoplasm or its transport into the vacuoles. *Nramp5*, a key transporter protein for cadmium uptake by the root system, is located at the plasma membrane and is highly expressed in the root maturation zone, responsible for the transport of cadmium from the outside into the root cells (Sasaki et al., 2012; Riaz et al., 2021). Silicon downregulates the expression of *Nramp5* in rice cells under cadmium toxicity (Ma et al., 2015). Treatment of rice suspension cells with silicon nanoparticles (SiNPs) reduces the expression levels of the genes encoding the cadmium transport proteins *OsLCT1* and *OsNramp5* (Cui et al., 2017). Due to the unique N-terminal sequence, the ATPase in plants is also named as HMAs. Among them, HMA3 is the main protein responsible for the transport of cadmium chelates into vesicles (Mohabubul Haque et al., 2022). The *OsHMA3* mediates the sequestration of cadmium in the root cell vacuole (Riaz et al., 2021). The transport of cadmium into vesicles may be an important mechanism for cadmium detoxification in plants (Li et al., 2021). Consistent with this, silicon upregulates the expression of *OsHMA3* in the root system (Cai et al., 2022). Cadmium toxicity increases the expression of four genes (*Nramp5*, *Nramp1*, *HMA2* and *HMA3*) that encode cadmium transporter proteins in rice. Exogenous application of silicon downregulates the expression of these four genes under cadmium stress (Chen et al., 2019). A homolog of *OsHMA3*, *OsHMA2*, localizes to the plasma membrane of root pericycle cells and is involved in the root-to-shoot translocation of cadmium; however, silicon downregulates the activity of *OsHMA2* (Shao et al., 2017). Located in the rice bast, *OsLCT1* mediates the transport of cadmium to the grain (Uraguchi et al., 2011). Silicon reduces the promotion of *OsLCT1* expression by cadmium (Greger et al., 2016). Thus, silicon application can minimize cadmium-induced damage to plants by reducing the uptake and transport of cadmium (Rizwan et al., 2012; Vaculík et al., 2012).

4 Coprecipitation

The coprecipitation of silicon and cadmium in soil reduces the availability of soil cadmium (Zhao et al., 2020). Silicon improves soil physical and chemical properties, reduces soil acidity (i.e., increases soil pH) and coprecipitates with heavy metals in the soil, forming metal-silicate complexes, to reduce the uptake of heavy metals by plants (Khan et al., 2021; Bhardwaj et al., 2022). Soluble silicates hydrolyze in soil solution and produce gel-like metasilicic acid, which binds heavy metals and changes the form of the metal from toxic to non-toxic (Bhat et al., 2019). Consistent with this result, the addition of SiNPs to cadmium-contaminated soil decreases the content of biologically effective cadmium in the soil (Hussain et al., 2019). Studies show that silicon-rich amendments fix copper, cadmium, and zinc in polymetallic acidic soils by increasing soil pH, and that metals are mainly in the form of silicates, phosphates, and hydroxides during amendment treatment exist (Gu et al., 2011). The use of silicon in the form of calcium silicate also changes the distribution of cadmium and zinc in soils, which are present in more stable forms, such as complexes with organic matter and crystalline iron oxide (Da Cunha et al., 2008). Among the four amendments tested on cadmium-contaminated soil, silicon showed the greatest reduction in the concentration of fast-acting cadmium (56.28%), greatly changed the soil microbial community (especially acidophilus and chiguria) and significantly reduced the bioavailability of cadmium to plants (Song et al., 2021).

The coprecipitation of silicon and metals in the cell walls of roots and leaves is an additional means of mitigating metal toxicity (Figure 1) (Wang et al., 2000; Ye et al., 2012; Cai et al., 2022). Cadmium and zinc co-precipitate with silicon in the cell walls of epidermal, ectodermal, endodermal, mesocolonial sheath and xylem cells in maize roots (Da Cunha and Do Nascimento, 2009). Similarly, the coprecipitation of silicon with cadmium in rice stems reduces the metal concentration in the leaves (Gu et al., 2011). Silicon coprecipitates with cadmium and zinc in the leaf cell cytoplasm and vesicles to form Si-metal complexes that contribute to cellular detoxification (Da Cunha and Do Nascimento, 2009). Increased silicon content in the cell wall may provide additional cadmium-binding sites (Cui et al., 2017). In the rice cell walls, electrostatic interactions between the silicon–hemicellulose matrix and cadmium cations, co-deposition of silicon with cadmium inhibit cadmium ion uptake and lead to *in vivo* detoxification of cadmium (Ma et al., 2015). This mechanism explains how silicon inhibits cadmium uptake and transport at the single-cell level. Moreover, silicon application may lead to an increase in root secretions, which can reduce heavy metal uptake by the roots because of the chelation of heavy metals (Keller et al., 2015).

5 Compartmentalization reaction

Vacuoles are important storage organelles for many ions, and high-level accumulation of toxic ions in vacuoles is an important strategy employed by plants to enhance heavy metal tolerance (Sterckeman and Thomine, 2020).

Phytochelatins (PCs) are a class of small biological molecules, with the structure (γ-Glu-Cys)n-Gly (n=2~11), synthesized by plant chelating peptide synthase (PCS) upon plant exposure to cadmium or other metals and oxygen-containing anions (Satofuka et al., 2001; Vestergaard et al., 2008). In plant cells, cadmium usually binds to PCs to form cadmium-PC complexes, which are then transported to and sequestered in vesicles to avoid further damage to cellular organelles (Shanmugaraj et al., 2019). Silicon increases the concentration of cadmium bound to PCs in rice (Cai et al., 2022). Similar result was also proved that the concentration of PCs with expression of *OsPCS1* in roots of cadmium-stressed was

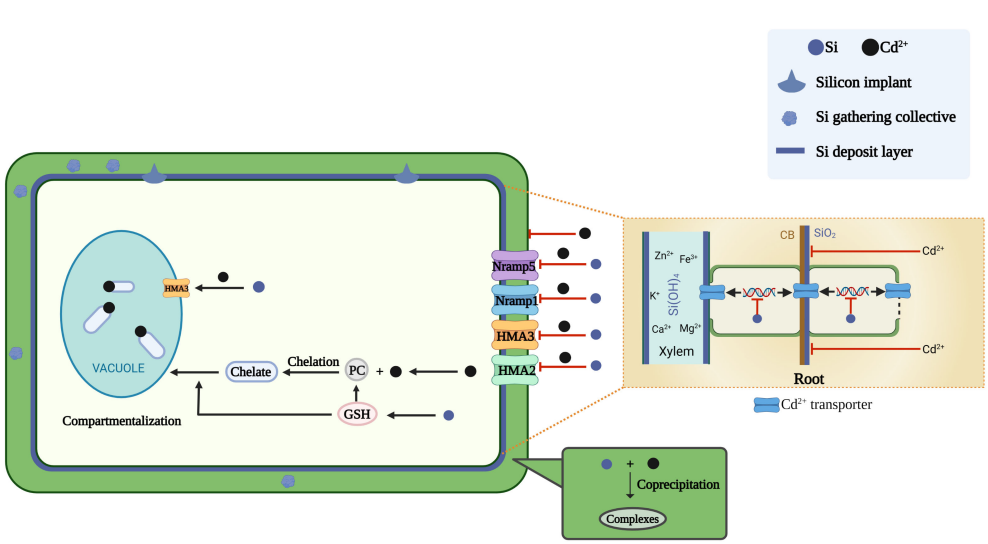

FIGURE 1
Mechanisms of silicon on reducing cadmium toxicity. The underlying mechanism includes (i) enhancing plant growth by promoting photosynthesis and nutrition uptake; (ii) reduction of cadmium accumulation due to silicon decreases cadmium uptake&transport gene expression and its deposition forms a physical barrier; (iii) the co-precipitation, compartmentalization of Cd^{2+}. Cartoon pictures were created with BioRender.com.

significantly induced when subjected to silicon treatment (Bari et al., 2020). PC2 and PC3 are the main PCs that synthesized in the cytoplasm of root cells after cadmium exposure (Volland et al., 2013). Silicon promotes the production of PC2 and PC3, thereby sequestering more PC-bound cadmium in the vacuole and limiting the migration of cadmium from roots to shoots (Wei et al., 2021). Glutathione both acts as a precursor for production of PCs which chelate cadmium in cells to form PC-cadmium complexes and also promotes the transport of these complexes into the vacuoles to reduce the cadmium concentration in the cytoplasm (Huang et al., 2021; Luyckx et al., 2021). Silicon increases glutathione and PC synthesis, allowing the plants to efficiently cope with oxidative stress through the improvement of cadmium sequestration on thiol groups in the roots (Luyckx et al., 2021). Stimulation of glutathione synthesis by silicon under cadmium stress was also found in rice (Farooq et al., 2016), sugar beet (Kabir et al., 2021), maize (Singh et al., 2019), wheat (Thind et al., 2020), and pepper (Kaya et al., 2020).

Heavy metals form complexes with PCs, which are transported to and sequestered in vesicles by ATP-binding cassette (ABC) transporters (Sharma et al., 2016). *OsHMA3*, a heavy metal ATPase, is a cadmium efflux protein to the vesicles and facilitates cadmium sequestration in the root vesicles (Sasaki et al., 2014). Rice under cadmium toxicity was mostly up-regulated in ABC transporter proteins after silicon treatment, which may contribute to the compartmentalization of cadmium in vesicles (Sun et al., 2022). The addition of SiNPs increases the expression of rice heavy metal ATPase 3 (*OsHMA3*) localized in the vacuole membrane and increases cadmium translocation into the vacuoles, thus reducing the effects of cadmium toxicity (Cui et al., 2017). Another heavy metal binding ligand, metallothioneins (MTs), have also been found in many plant species (Cobbett and Goldsbrough, 2002).

6 Silicon improves plant mineral nutrient supply

High concentrations of cadmium reduce the ability of plants to absorb and transport nutrients and disrupt mineral metabolism, leading to nutrient deficiencies (Luyckx et al., 2021). Cadmium competes with mineral nutrient ions for the same transport system, resulting in the shortage of nutrients required for plant growth and development (Alcántara et al., 1994; Sarwar et al., 2010; Nazar et al., 2012). Silicon facilitates the uptake and utilization of plant nutrients was summarized in Table 2. This may be one of the reasons why silicon promotes plant growth under cadmium toxicity (Pilon et al., 2013; Hernandez-Apaolaza, 2014). Exogenous application of silicon promotes the uptake of nutrients by plant roots, including mineral elements such as Zn, Fe, Mn, Ca, Mg, P, and K, thereby offsetting the shortage caused by cadmium and thus promoting plant growth (Keller et al., 2015; Wang et al., 2015; Rahman et al., 2021c). The effect of silicon on the concentration of mineral elements varies depending on the crop type, plant organism and type of mineral

TABLE 2 Effect of silicon on the supply of mineral nutrients to plants under cadmium poisoning.

Species	Silicon concentration	Cadmium concentration	Effect	Reference
Wheat	1 mg kg^{-1} $CaSiO_3$	10 mg kg^{-1} $CdSO_4{\cdot}8H_2O$	Applied Si increases Zn, Fe, Cu content in roots and grain.	(Farooqi et al., 2022)
Bean	20 mg L^{-1} SiNPs	1, 1.5 and 2 mM $CdCl_2$	Silicon enhancing K^+ accumulation is beneficial to reduce stress effect.	(Koleva et al., 2022)
Hemp	2 mM H_2SiO_3	20 μM $CdCl_2$	Cd decreased Fe and K concentration in the leaves. The addition of Si strongly increases leaf Fe content.	(Luyckx et al., 2021)
Wheat	1 and 3 mM Na_2SiO_3	50 and 200 μM $CdCl_2$	Silicon increases the of N, P, K, Ca, Mg, and Zn.	(Rahman et al., 2021c)
Wheat	1.5 and 3 mM Na_2SiO_3	10 and 25 mg kg^{-1} $CdCl_2$	Cadmium poisoning reduced Fe^{2+}, Zn^{2+}, Mg^{2+} contents in roots and shoots, and silicon application increased Fe^{2+}, Zn^{2+}, Mg^{2+} contents in roots and shoots.	(Thind et al., 2021a)
Ajwain	1.5 and 3 mM K_2SiO_3	1.5, 3 mM $CdCl_2$	Si increases the concentration of Fe, Mg, K and Ca.	(Javed et al., 2020)
Pepper	2.0 mM Na_2SiO_3	0.1 mM $CdCl_2$	Cadmium significantly reduces K and Ca concentration, but silicon promotes the uptake of these two elements, keeping their levels roughly the same as the control (no-stress).	(Kaya et al., 2020)
Wheat	1 mM Na_2SiO_3	50, 100 and 200 μM $CdCl_2$	N and K concentrations are reduced by cadmium, which is alleviated by silicon.	(Rahman et al., 2021a)
Wheat	3 mM Na_2SiO_3	25 mg kg^{-1}	Cadmium poisoning reduces Mg^{2+}, Zn^{2+}, Fe^{2+} in roots and Mg^{2+}, Zn^{2+} in the shoots, while silicon application increases Mg^{2+}, Zn^{2+}, Fe^{2+} in roots and Mg^{2+}, Zn^{2+} in the shoots under cadmium poisoning.	(Thind et al., 2020)
Maize	10 μM Na_2SiO_3	100 μM $CdCl_2$	Cadmium poisoning reduces Ca contents in seedlings, and silicon application increases Ca contents. Cadmium poisoning increases S contents in seedlings, and reduces S contents in seedlings.	(Singh et al., 2019)

(Continued)

TABLE 2 Continued

Species	Silicon concentration	Cadmium concentration	Effect	Reference
Pigeonpea	300 mg kg^{-1} K_2SiO_3	25 and 50 mg kg^{-1} $CdSO_4$	Cadmium toxicity causes a significant decrease in N, P, K, and Mg content in plant leaves, and the addition of silicon to cadmium-poisoned plants increases N, P, K, and Mg content.	(Garg and Singh, 2018)
Cowpea	1.25 and 2.50 mM $Na_2SiO_3{\cdot}9H_2O$	500 µM $CdCl_2$	Cadmium reduces the contents of macronutrients (P, K, Ca, Mg and S) and micronutrient (Zn, Cu, Fe, Mn and Mo). However, 500 M cadmium + 2.50 mM silicon treatment significantly increases all of the above elements.	(Pereira TS et al., 2018)
Gladiolus grandiflora L.	200 mg L^{-1} Si	50 mg kg^{-1} $CdSO_4{\cdot}8H_2O$	Cadmium poisoning reduces S, Mn, Ca, Mg, and K contents in roots and shoots and silicon application increased S, Mn, Ca, Mg, and K contents in roots and shoots.	(Zaheer et al., 2018)
Peas	1.8 mM H_4SiO_4	20 µM $CdSO_4$	Si reduces Cd translocation in shoots through the regulation of Fe transport.	(Rahman et al., 2017)
Rice	2.5 mM Nano-silicon	20 µM Cd^{2+}	Nanosilicon increase Mg, Zn, Cu, Mn and root Fe but reduce Ca, Mn, Cu and shoot Fe	(Wang et al., 2015)

element. In poplar callus cells, silicon attenuates plasma membrane damage caused by cadmium toxicity and promotes the uptake and transport of nutrients including Ca, K, Mg, P, Fe, Zn, and Mn after 3 and 9 weeks of cadmium treatment (Kučerová et al., 2020). In hemp, cadmium stress increases the content of Ca, P and S in stems and leaves and that of Fe in roots but decreases the content of Fe in leaves. Additionally, silicon greatly increases Fe concentration in the leaf and alleviates cadmium-induced reduction in Ca concentration in the root and cadmium-induced accumulation of P in the shoot (Luyckx et al., 2021). On the contrary, silicon has no significant impact on S accumulation in the shoot under cadmium stress (Luyckx et al., 2021). Foliar application of silicon increases the concentration of N and P; but decreases the concentration of Na. Because silicon has no effect on the concentration of K, treatment with silicon decreases the ratio of K^+/Na^+ (Rady et al., 2019). In ajwain, cadmium decreases Fe, Mg, K and Ca both in shoot and root (Javed et al., 2020).

Although some studies have been conducted to reveal the effect of silicon on plant mineral elements by silicon under cadmium stress, most of them only demonstrated the results, lacking the exploration of the deep mechanism analysis. Thus, how silicon regulates the uptake of mineral elements and how these mineral elements enhance cadmium resistance in plants remain largely unclear.

7 Silicon-induced antioxidant defenses

The concentration of ROS in plant cells is normally low and poses little danger to cellular functioning. However, upon exposure to heavy metal toxicity, ROS concentrations increase dramatically, triggering a series of physiological and biochemical changes, which eventually cause yield reduction and even plant death. Cadmium increases the production of ROS in plants and causes oxidative stress (Schutzendubel et al., 2001; Farooq et al., 2013). Under cadmium toxicity, large amounts of ROS, including singlet oxygen ($^1O_2$), superoxide ion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-), accumulate in plant cells (Gallego et al., 2012; Liu et al., 2013b). Plants synthesize antioxidant enzymes, such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX), to cope with the adverse effects of high ROS accumulation under cadmium stress (Li et al., 2018). Silicon can enhance antioxidant capacity of plants (Figure 2). Silicon dioxide nanoparticles stimulate the antioxidant defense system in wheat and rice (Tripathi et al., 2017). The application of silicon increases the activities of SOD, POD, CAT, APX and GPX in different wheat cultivars under cadmium toxicity and the degree of enhancement in antioxidant enzyme activity is higher in cadmium-tolerant cultivars than in cadmium-sensitive cultivars (Naeem et al., 2018; Hussain et al., 2019). Foliar spray of silicon on rice plants under cadmium toxicity increases the branch POD and SOD activities and decreases CAT activity (Wang et al., 2015). Similarly, in ginger, silicon increases SOD, POD and CAT activities under cadmium toxicity (Chen et al., 2020). Exogenous silicon treatment increases SOD, CAT and APX activities in cucumber leaves. However, in tomato leaves, silicon increases only SOD activity and decreases other antioxidant enzyme activities under cadmium toxicity (Wu et al., 2015). Moreover, silicon promotes the rapid accumulation of polyamines, scavenges free radicals and prevents heavy metal-induced oxidative damage (Das et al., 2022). Spray application of silicon onto the leaf surface enhances the production of glutamyl kinase (the first committed enzyme in the proline biosynthesis pathway) and decreases the production of proline oxidase (responsible for the denaturation of proline molecules) to promote proline synthesis and help plants to cope with oxidative stress (Elnaz et al., 2021).

By contrast, some studies demonstrated that silicon reduces antioxidant enzyme activity under cadmium stress. For instance, compared to cadmium treatment alone, the presence of silicon reduces the SOD, POD, CAT and APX activity in *Solanum nigrum* L. (Liu et al., 2013b). In wheat, cadmium stress increased SOD, CAT and POD activities by 77.18%, 76.95% and 108.33%, respectively, compared with the control, whereas foliar application of 4.50 mM silicon improved the SOD, POD and CAT activities by 23.91%, 32.01% and 69.76%, respectively (Heile et al., 2021). These contrasting results may be caused by differences in plant species, cultivars, age, silicon source (silicon or nano-silicon), treatment time and experimental conditions (Table 3).

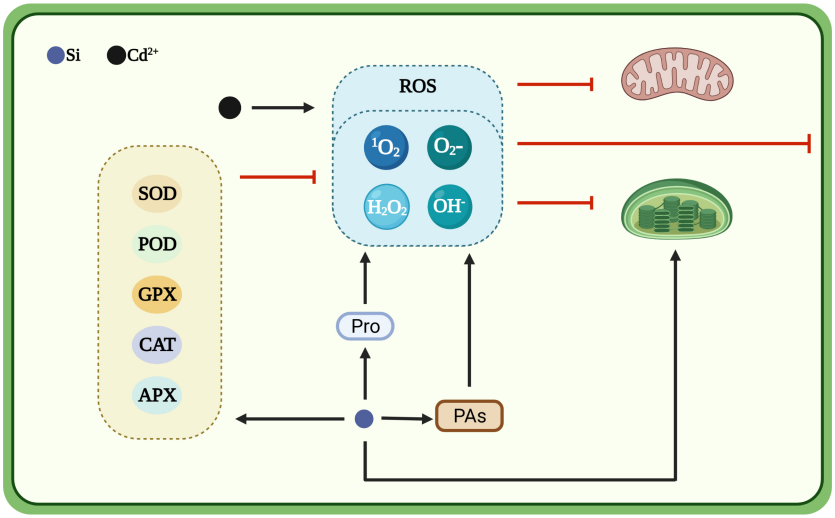

FIGURE 2
Silicon-induced antioxidant defense system mitigates the toxic effects of cadmium. Cartoon pictures were created with BioRender.com.

8 Silicon adjusts the water balance

Water is the most important constituent of living organisms. Every plant cell requires water for survival. Without water, plants cannot perform photosynthesis (the process of food production), respiration nutrient translocation to different plant parts. Adverse environmental conditions such as drought, salt, evaporation, chilling and heavy metal toxicity can disturb plant water balance, which in turn inhibits plant growth (Bray, 1997; Tchounwou et al., 2012).

Cadmium is mainly present in the soil as the divalent cation (Cd^{2+}), which is absorbed by the root system and then translocated

TABLE 3 Effect of silicon on plant antioxidant system under cadmium poisoning.

Species	Silicon concentration	Cadmium concentration	Results	Reference
Maize	6 mM K_2SiO_3	500 μM $CdCl_2$	SOD, APX, POD↑	(Saleem et al., 2022)
Rice	2.5 mM SiNPs	50 μM $CdCl_2{\cdot}2.5H_2O$	APX, GSH↑	(Riaz et al., 2022)
Pepper	2.0 mM Na_2SiO_3	0.1 mM $CdCl_2$	GSH, ASA, SOD, POD, CAT↑	(Kaya et al., 2020)
Maize	10 μM Na_2SiO_3	100 μM $CdCl_2$	SOD, APX, CAT, GR, DHAR, MDHAR, ASA, GSH↑	(Singh et al., 2019)
Rape	0.6 mM $Na_2SiO_3{\cdot}9H_2O$	50 μM $CdCl_2{\cdot}2.5H_2O$	SOD, CAT, POD↑	(Zong et al., 2022)
Basil	1 and 2 mM Na_2SiO_3	25 and 50 mg kg^{-1} Cadmium nitrate	SOD, CAT, APX↑; Proline↓	(Gheshlaghpour et al., 2021)
Isatis cappadocica Desv	0.5, 1and 2 mM Na_2SiO_3	600 μM $CdCl_2$	GST, GR↑	(Azam et al., 2021)
Lettuce	1 mM Si solution	1 mM $CdCl_2$	SOD, CAT↑; APX↓	(Pereira et al., 2021)
Wheat	3 mM SiNPs	25 mg kg^{-1} $CdCl_2$	SOD, POD, APX, CAT↑	(Thind et al., 2021b)
Ajwain	1.5 and 3 mM K_2SiO_3	1.5 and 3 mM $CdCl_2$	CAT, APX↑	(Javed et al., 2020)
Wheat	1 and 3 mM Na_2SiO_3	50 and 200 μM $CdCl_2$	CAT, POD, SOD↑	(Rahman et al., 2021b)
Wheat	3 mM Na_2SiO_3	25 mg kg^{-1} Cd^{2+}	SOD, POD, CAT, APX, ASA, GSH↑	(Thind et al., 2020)
Wheat	1 mM Na_2SiO_3	50, 100 and 200 μM $CdCl_2$	CAT, SOD, POD, Proline↑	(Rahman et al., 2021b)

(Continued)

TABLE 3 Continued

Species	Silicon concentration	Cadmium concentration	Results	Reference
Wheat	300, 600, 900 and 1200 mg kg^{-1} SiNPs	7.38 mg kg^{-1} Cd^{2+}	SOD, POD↑	(Ali et al., 2019)
Rice	1.5 mM $NaSiO_3·9H_2O$	100 µM $CdCl_2$	SOD, POD, CAT↑	(Chen et al., 2019)
Wheat	1 g kg^{-1} Organosilicon and Sodium silicate	2.82 mg kg^{-1} Cd^{2+}	POD↑; SOD, CAT, GSH↓	(Huang et al., 2019)
Wheat	300, 600, 900 and 1200 mg L^{-1} SiNPs	7.38 mg kg^{-1} Cd^{2+}	SOD, POD, CAT↑	(Hussain et al., 2019)
Wheat	3 mM $K_2SiO_3.nH_2O$	2 mM Cd^{2+}	Proline, AsA, GSH, SOD, CAT, POD↑	(Howladar et al., 2018)
Rice	1, 2, 4 and 6 g kg^{-1} $Na_2SiO_3·9H_2O$	3.51 mg kg^{-1}	SOD, POD, APX, CAT↑	(Li et al., 2018)
Pfaffia glomerata (Spreng.)	2.5 mM Na_2SiO_3	50 µM $CdCl_2$	SOD, POD↑	(Pereira TS et al., 2018)
Cowpea	1.25 and 2.50 mM $Na_2SiO_3·9H_2O$	500 µM $CdCl_2$	SOD, CAT, APX. POX↑	(Pereira AS et al., 2018)
Wheat	1 mM $Na_2SiO_3·9H_2O$	0, 5 and 20 µM $CdCl_2·3/2H_2O$	SOD, POD↑	(Shi et al., 2018)
Cabbage	5 µM Na_2SiO_3	1, 5 µM $CdCl_2$	SOD, APX, CAT↑	(Wu et al., 2018)
Gladiolus grandiflora L.	200 mg L^{-1} Si	50 mg kg^{-1} $CdSO_4·8H_2O$	SOD, POD, CAT, APX↑	(Zaheer et al., 2018)
Arabidopsis thaliana	400 mg kg^{-1} Na_2SiO_3	100 mM Cd^{2+}	APX, CAT, GR↑	(Carneiro et al., 2017)
Brassica napus L	1 mM SiO_2	0.5 and 1.0 mM $CdCl_2$	AsA, GSH, APX, MDHAR, DHAR, GR, CAT, Gly I, Gly II↑	(Hasanuzzaman et al., 2017)
Peas	1.8 mM H_4O_4Si	20 µM $CdSO_4$	CAT, POD, GR, SOD↑	(Rahman et al., 2017)
Alfalfa	1 mM K_2SiO_3	1 mM $CdCl_2$	CAT, APX, SOD, Methionine and Proline↑ GR↓	(Kabir et al., 2016)
Rice	200 µM K_2SiO_3	2 mM $Cd(NO_3)_2$	CAT↑; SOD, GPX, APX↓	(Srivastava et al., 2015)
Rice	2.5 mM Nano-silicon	20 µM Cd^{2+}	GSH, SOD, Shoot POD, Root CAT↑ Root POD, Shoot CAT↓	(Wang et al., 2015)
Tomato Cucumber	0.5 mM (cucumber) and 2 mM (tomato) $Na_2SiO_3·9H_2O$	100 µM $CdCl_2$	SOD, CAT, GR(tomato cucumber), APX (tomato)↑; APX(cucumber)↓	(Wu et al., 2015)
Cotton	1 mM Na_2SiO_3	1 and 5 mM $CdCl_2$	SOD, GPX, CAT, APX↑	(Farooq et al., 2013)
Solanum nigrum L	1 mM Na_2SiO_3	100 mM $CdCl_2$	SOD, POD, CAT, APX↓	(Liu et al., 2013a)
Maize	0.08 mM Na_2SiO_3	5 and 10 µM $Cd(NO_3)_2·4H_2O$	SOD, POX↑; CAT↓	(Lukačová et al., 2013)
Peanut	1.8 mM Si	200 µM Cd^{2+}	SOD, POD, CAT↑	(Shi et al., 2010)

"↑"="increased", "↓"="decreased".

to the shoot through the xylem along the transpiration stream (Sterckeman and Thomine, 2020). Because transpiration pull is the main driving force for cadmium transport from the root system to the aboveground parts, lower transpiration rate is likely to reduce cadmium accumulation in the leaf (Uraguchi et al., 2009; Zulfiqar et al., 2021). The application of silicon significantly reduces stomatal conductance and subsequently constrains the transpiration rate, which reduces cadmium transport from roots to shoots in wheat (Naeem et al., 2018). Similarly, silicon reduces stomatal conductance and transpiration rate in *Cannabis sativa* under cadmium stress (Luyckx et al., 2021). In rice, cadmium significantly reduces transpiration, and the reduction in transpiration increases further upon the addition of silicon. Interestingly, the transpiration rate of plants treated with 0.2 mM silicon is significantly lower than that of plants treated with 0.6 mM silicon (Nwugo and Huerta, 2008). Rizwan et al. (2016) indicated that silicon-induced reduction in transpiration would reduce cadmium translocation to shoots. Additionally, silicon application

decreases transpiration rate by 65% and 42% in - cadmium and + cadmium plants, respectively (Nwugo and Huerta, 2011).

Contrary to the above result, there are accumulating experiments proved that under cadmium stress silicon enhances gas exchange indices (especially represented by transpiration rate and stomatal conductance) in rice (Table 4; Gao et al., 2018; Li et al., 2018; Rehman et al., 2019; Rizwan et al., 2019; Sohail et al., 2019; Huang et al., 2021; Mapodzeke et al., 2021), wheat (Alzahrani et al., 2018; Shi et al., 2018; Ali et al., 2019; Hussain et al., 2019), peas (Jan et al., 2018), beans (Rady et al., 2019; Ahmad et al., 2021; El-Saadony et al., 2021), maize (Sohail et al., 2019), gladiolus (Zaheer et al., 2018), pepper (Kaya et al., 2020), ajwain (Javed et al., 2020), cabbage (Yang et al., 2018). In comparison with control (non-stressed) conditions, cadmium stress conditions reduce photosynthetic rate (37.29%), transpiration rate (37.28%), stomatal conductance (38.09%) and chlorophyll content (14.13%). Silicon supplementation improves plant tolerance to cadmium stress. The greatest influence on all physio-biochemical attributes was noticed in plants supplemented with 4.50 mM silicon under cadmium stress. In the presence of 4.50 mM silicon, the most promising level, increases in the photosynthetic rate (45.77%), rate of transpiration (38.60%), stomatal conductance (42.85%), chlorophyll contents (45.77%) and water use efficiency (7.77%) compared with the relevant control (Heile et al., 2021). In rice, foliar spray of silicon reduces the accumulation of cadmium in leaves but increases the transpiration rate and stomatal conductance of leaves, compared with the control, under cadmium stress. Furthermore, structural equation modeling indicated that transpiration rate and

TABLE 4 Effect of silicon on the water balance under cadmium poisoning.

Species	Silicon concentration	Cadmium concentration	Effect of silicon on, Tr, Gs, RWC and WUE	Reference
Peas	0.5, 1 and 1.5 mM Na_2SiO_3 and K_2SiO_3	20 mg kg^{-1} $CdCl_2$	Tr, Gs, RWC↑	(Batool et al., 2022)
Wheat	1 mM Na_2SiO_3	100 and 200 µM $CdSO_4{\cdot}8H_2O$	RWC↑	(Saber et al., 2022)
Beans	2 mM Na_2SiO_3	75 mg kg^{-1} Cd^{2+}	Tr, Gs, RWC↑	(Ahmad et al., 2021)
Summer savory	0.75, 1.5 and 2.25 mM SiNPs	10 and 20 mg kg^{-1} $CdCl_2$	RWC↑	(Elnaz et al., 2021)
Beans	2.5, 5 mM	18.6 mg kg^{-1} Cd^{2+}	Tr, Gs, RWC↑	(El-Saadony et al., 2021)
Wheat	1.5, 3.0 and 4.5 mM silicon compounds (K_2SiO_3 and $CaSiO_3$)	20 mg kg^{-1} $CdCl_2$	Tr, Gs, RWC↑	(Heile et al., 2021)
Rice	1.5 mM $Na_2SiO_3{\cdot}9H_2O$	100 µM $CdCl_2{\cdot}2H_2O$	Tr, Gs↑	(Huang et al., 2021)
Hemp	2 mM H_2SiO_3	20 µM $CdCl_2$	Tr, Gs↓	(Luyckx et al., 2021)
Rice	5, 15 µM $Na_2SiO_3{\cdot}9H2O$	15 µM $CdCl_2$	Tr↑	(Mapodzeke et al., 2021)
Wheat	1, 3 mM Na_2SiO_3	50, 200 µM $CdCl_2$	RWC↑	(Rahman et al., 2021a)
Ajwain	1.5, 3 mM K_2SiO_3	1.5, 3 mM $CdCl_2$	Tr, Gs↑	(Javed et al., 2020)
Pepper	2 mM Na_2SiO_3	0.1 mM $CdCl_2$	RWC↑	(Kaya et al., 2020)
Wheat	300, 600, 900 and 1200 mg kg^{-1} SiNPs	7.38 mg kg^{-1} Cd^{2+}	Tr, Gs↑	(Ali et al., 2019)
Sunflower	100 mg kg^{-1} Na_2SiO_3	20, 40 mg kg^{-1} $CdSO_4$	RWC↑	(Ashraf et al., 2019)
Wheat	300, 600, 900 and 1200 mg L^{-1} SiNPs	7.38 mg kg^{-1} Cd^{2+}	Tr, Gs↑	(Hussain et al., 2019)
Beans	6 mM $K_2SiO_3{\cdot}nH_2O$	1.5 mM $CdCl_2$	Tr, RWC↑	(Rady et al., 2019)
Rice	0.03% w/w K_2SiO_3	0.52 mg kg^{-1} $CdSO_4{\cdot}8/3H_2O$	Tr, Gs↑	(Rehman et al., 2019)
Rice	5, 10, 15 and 20 mg L^{-1} Nanosilica	7.86 mg kg^{-1} Cd^{2+}	Tr, Gs↑	(Rizwan et al., 2019)
Rice, Maize	150 mg kg^{-1} SiO_2	6.81 mg kg^{-1} Cd^{2+}	Tr, Gs↑	(Sohail et al., 2019)
Wheat	2, 4 and 6 mM $K_2SiO_3{\cdot}nH_2O$	2 mM Cd^{2+}	Tr, Gs, RWC↑	(Alzahrani et al., 2018)
Rice	2.5 mM Si	0.3 mg kg^{-1} Cd^{2+}	Tr, Gs↓	(Gao et al., 2018)

(Continued)

TABLE 4 Continued

Species	Silicon concentration	Cadmium concentration	Effect of silicon on, Tr, Gs, RWC and WUE	Reference
Wheat	3 mM $K_2SiO_3{\cdot}nH_2O$	2 mM Cd^{2+}	Tr, Gs, RWC, WUE↑	(Howladar et al., 2018)
Beans	2 mM Na_2SiO_3	150 mg L^{-1} $CdSO_4{\cdot}8H_2O$	Tr, Gs, RWC↑	(Jan et al., 2018)
Rice	1, 2, 4 and 6 g kg^{-1} $Na_2SiO_3{\cdot}9H_2O$	3.51 mg kg^{-1} Cd^{2+}	Tr↑	(Li et al., 2018)
Wheat	0.6 mM $SiO_2{\cdot}nH_2O$	15 mM $CdCl_2{\cdot}H_2O$	Tr, Gs↓	(Naeem et al., 2018)
Wheat	1 mM $Na_2SiO_3{\cdot}9H_2O$	5, 20 μM $CdCl_2{\cdot}3/2H_2O$	Tr, Gs↑	(Shi et al., 2018)
Cabbage	1.2 mM $Na_2SiO_3{\cdot}9H_2O$	50 μM Cd^{2+}	Tr, Gs↑	(Yang et al., 2018)
Gladiolus	200 mg L^{-1} Si	50 mg kg^{-1} $CdSO_4{\cdot}8H_2O$	Tr, Gs↑	(Zaheer et al., 2018)
Cotton	1 mM Na_2SiO_3	1 and 5 mM $CdCl_2$	Tr, Gs, WUE↑	(Farooq et al., 2013)

"↑"="increased", "↓"="decreased".

stomatal conductance have negative effects on cadmium concentration in rice (Gao et al., 2018). Compared with the control (no-stress condition), cadmium stress reduces the transpiration rate and stomatal conductance of wheat leaves, but external silicon application significantly alleviates the inhibitory effect of cadmium on these indicators and subsequently enhances the water use efficiency (WUE) of plants (Howladar et al., 2018). Notably, in this study, relative leaf water content (RWC), a common indicator of leaf water balance, was also enhanced by silicon under stress (Howladar et al., 2018). Similar results have also been reported in bean (Rady et al., 2019; Ahmad et al., 2021; El-Saadony et al., 2021), pea (Jan et al., 2018) and pepper (Kaya et al., 2020). Silicon addition maintains RWC in wheat cells and tissues under cadmium poisoning (Alzahrani et al., 2018; Heile et al., 2021). The addition of silicon significantly increases RWC to maintain cell expansion pressure and protects plants from wilting and cell relaxation to mitigate plant cadmium toxicity (Rahman et al., 2021a). In addition, and more importantly, an increase in the leaf water content by silicon could dilute the cadmium concentration, subsequently reduce cadmium toxicity in plants (Kaya et al., 2020).

Plant leaf water content depends on the dynamic balance between water loss from leaves and water uptake by roots; thus, changes in both these processes affect the plant water status. Transpiration is the primary mode of water loss from shoots (Bernacchi and Vanloocke, 2015; Wang et al., 2018). As mentioned above, silicon-induced decrease in transpiration not only reduces the translocation of cadmium from roots to leaves but also reduces leaf water loss, resulting in higher RWC than the cadmium treatment (Chen et al., 2018).

In addition to water loss, water uptake also influences the RWC (Steudle, 2000; Liu et al., 2014). The RWC of wheat significantly decreases under drought, salinity and cadmium stresses. However, the presence of silicon significantly reduces the destructive effects of the above stresses on RWC. According to these data, we can infer that the mitigation effect of silicon on RWC is similar in the three stress conditions. In other words, the effect of silicon on leaf water content is independent of the stress type (Alzahrani et al., 2018). Therefore, we can hypothesize that the mechanism of silicon on enhancing water balance revealed in other stresses may also apply to cadmium toxicity. Liu et al. (2014) revealed that silicon application maintains the water balance in sorghum by elevating root water uptake under stress conditions. This phenomenon has been confirmed in other studies (Liu et al., 2015; Zhu et al., 2015; Chen et al., 2016).

Water channel proteins play a major role in regulating root water uptake and shoot water transport under stress (Hachez et al., 2012). Silicon-mediated increase in the expression of the plasma membrane intrinsic protein water channel protein (*PIP*) gene is associated with an increase in root hydraulic conductivity and water uptake (Rios et al., 2017). An effect of silicon on aquaporin gene expression has been observed in sorghum (Liu et al., 2014), cucumber (Zhu et al., 2015) and barley (Celikkol and Erkan, 2016). In tobacco, we recently observed that the expression of *NtPIP* genes was strongly enhanced under cadmium stress (unpublished data). In addition to the expression of aquaporin genes, the content of aquaporin proteins is also influenced by silicon. In rapeseed plants under drought stress, silicon application promotes the expression of *BnPIP1*, *BnPIP2-1-7* and *BnTIP1;1* and the accumulation of aquaporins (Gao et al., 2006; Saja-Garbarz et al., 2022). These findings show that water channel protein transport activity can be regulated at the post-transcriptional level. The effect of silicon on plant water balance under cadmium stress is summarized in Figure 3.

9 Conclusions and perspectives

There is increasing evidence that silicon can improve plant growth under exposure to cadmium toxicity. The reported mechanisms include complexation and coprecipitation of silicon with cadmium in different plant organs, compartmentalization of cadmium in different subcellular organelles, structural alteration of the plant, reduction of cadmium content, regulation of cadmium

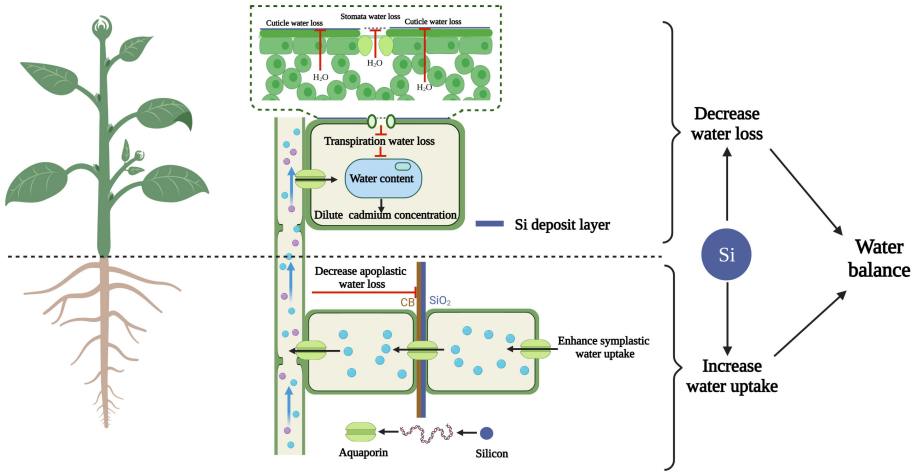

FIGURE 3
Silicon-diluted cadmium concentration by maintaining the water balance (increasing root water uptake and decreasing leaf water loss). Cartoon pictures were created with BioRender.com.

transporter gene expression, improvement of plant mineral nutrient supply (Figure 1), enhancement of ROS scavenging (Figure 2) and maintenance of leaf water balance (Figure 3).

However, the molecular mechanism underlying the effect of silicon on plant reactions remains unknown. Further research is needed to determine whether silicon directly or indirectly participates in plant physiological responses or gene expression regulation under cadmium stress. An increasing number of studies show that the function of silicon is mediated by signaling messengers, such as plant hormones, ROS and Ca^{2+}. Thus, the crosstalk between silicon and signaling messengers may constitute an important research focus to elucidate the mechanism of silicon-mediated increase in cadmium tolerance in plants.

Author contributions

LH: Writing-original draft; SJ: Visualization, Writing-original draft; YZ: Writing-review & editing; XW: Methodology; LZ: Writing-review & editing; PL: Conceptualization, Writing review & editing, Funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Muhammad Musa Khan,
Zhejiang University, China

REVIEWED BY
Tara Chand Yadav,
Indian Institute of Technology Roorkee, India
Tanushri Chatterji,
Institute of Management Studies Ghaziabad (University Courses Campus), India
Vijay Kumar,
University of Oxford, United Kingdom

*CORRESPONDENCE
Ashish Patel
uni.ashish@gmail.com
Madhvi Joshi
jd1-gbrc@gujarat.gov.in
Snehal Bagatharia
drsnehal.bagatharia@gmail.com

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Evaluation of the growth-inducing efficacy of various *Bacillus* species on the salt-stressed tomato (*Lycopersicon esculentum* Mill.)

Anil Patani[1], Dharmendra Prajapati[1], Daoud Ali[2], Haresh Kalasariya[3], Virendra Kumar Yadav[4], Jigna Tank[5], Snehal Bagatharia[6]*, Madhvi Joshi[7]* and Ashish Patel[4]*

[1]Department of Biotechnology, Smt. S. S. Patel Nootan Science and Commerce College, Sankalchand Patel University, Visnagar, Gujarat, India, [2]Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia, [3]Centre for Natural Products Discovery, School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Liverpool, United Kingdom, [4]Department of Life Sciences, Hemchandracharya North Gujarat University, Patan, Gujarat, India, [5]Department of Biosciences, Saurashtra University, Rajkot, Gujarat, India, [6]Gujarat State Biotechnology Mission (GSBTM), Udyog Bhavan, Gandhinagar, Gujarat, India, [7]Gujarat Biotechnology Research Centre (GBRC), Gandhinagar, Gujarat, India

Plants are affected by salt stress in a variety of ways, including water deficiency, ion toxicity, nutrient imbalance, and oxidative stress, all of which can cause cellular damage or plant death. Halotolerant plant growth-promoting rhizobacteria (PGPR) could be a viable alternative for tomato plants growing in arid and semi-arid environments. The aim of this research was to isolate halotolerant plant growth promoting *Bacillus* sp. to promote tomato (*Lycopersicon esculentum* Mill.) growth and salt stress resistance. 107 PGPR strains were isolated from the rhizospheres of 'Kesudo' (*Butea monosperma* Lam.), 'Kawaria' (*Cassia tora* L.), and 'Arjun' (*Terminalia arjuna* Roxb.) plants to test their plant growth promoting abilities, including indole-3-acetic acid, phosphate solubilization, siderophore production, and ACC deaminase activity. Five bacterial strains (*Bacillus pumilus* (NCT4), *Bacillus firmus* (NCT1), *Bacillus licheniformis* (LCT4), *Bacillus cereus* (LAT3), and *Bacillus safensis* (LBM4)) were chosen for 16S rRNA on the basis of PGPR traits. Compared to PGPR untreated plants, tomato plants developed from PGPR-treated seeds had considerably increased germination percentage, seedling growth, plant height, dry weight, and leaf area. As comparison to PGPR non-inoculated plants, salt-stressed tomato plants treated with PGPR strains had higher levels of total soluble sugar, proline, and chlorophyll as well as higher levels of SOD, CAT, APX, and GR activity. PGPR-inoculated salt-stressed tomato plants had lower MDA, sodium, and chloride levels than non-inoculated plants. In addition, magnesium, calcium, potassium, phosphorus, and iron levels were higher in PGPR treated plants when subjected to salt stress. These results indicate that halotolerant PGPR strains can increase tomato productivity and tolerance to salt stress by removing salt stress's negative effects on plant growth.

KEYWORDS

PGPR, salt stress, tomato, plant growth promotion, antioxidant enzymes

1 Introduction

Any government has significant difficulties as a result of the rising population and food demand, which transfers the focus to agricultural practices and improving yield improvement. Different agro-ecosystems' varying climatic conditions, edaphic elements, farming practices, and management strategies are the main determinants of increased crop productivity, which is highly intricate (Shah and Wu, 2019). Crop productivity is hindered by a number of abiotic conditions, including temperature, salinity stress, drought, soil pH, heavy metals, and the use of pesticides and chemical fertilizers (Wei et al., 2020). Salinity stress is considered a serious danger to agricultural production among all of these (Isayenkov and Maathuis, 2019). In arid and semi-arid areas, soil salinity is a major environmental issue that causes imbalanced osmotic stress and reduced plant growth (Cicek and Çakirlar, 2002). Salinity can affect all vegetable crops at varying degrees (Shannon and Grieve, 1998). Salinity can have varied effects on all vegetable crops, reducing crop growth and output *via* altering morphological and physiological processes (Shahbaz et al., 2012). Vegetable growth is impacted by salt stress because of the osmotic or water-deficit effect, toxic salt buildup in shoots, nutritional imbalances, or a combination of these factors (Läuchli and Grattan, 2017).

Many aspects of plant metabolism are affected by salinity stress, and as a consequence, yield and growth are diminished. A high salt content in a soil solution may hinder plant growth either through osmotic inhibition of root water uptake or through particular ion effects. Salinity raises the uptake of Na^+, which reduces the uptake of Ca^{2+} and K^+ (Yildirim et al., 2006). Surplus Na^+ can cause metabolic changes in processes that require low Na^+ and high K^+ or Ca^{+2} for optimal function (Marschner, 1995). Cl^- uptake and buildup may impair photosynthetic function by reducing nitrate reductase activity (Xu et al., 2000). When a cell's ability to accumulate salts is depleted, salts accumulate in the intercellular space, causing cell dehydration and demise (Sheldon et al., 2017). Higher saline levels cause the growth rate of the leaf area to slow down, as does the rate of leaf production and leaf size, ultimately resulting in the death of the plant (Suárez and Medina, 2005).

Among the many methods and techniques employed to lessen the detrimental effects of salinity stress on crop production are plant genetic engineering, the use of salt-resistant varieties, organic matter conditioners, and salt stress mitigation substances (Zhang et al., 2000). Salinity, however, is a challenging issue that scientists are working to resolve by creating more practical and affordable solutions that are straightforward to use. These helpful bacteria cause biochemical and morphological changes in the plants once they are inoculated with them, increasing the plants' resistance to abiotic stress (Kerbab et al., 2021). Due to the rise of more extreme climatic circumstances, inoculating plants with PGPB (plant growth promoting bacteria) can be an efficient way to enhance plant growth by increasing plant resilience to abiotic stresses (Redondo-Gómez et al., 2021). Plant growth promoting rhizobacteria (PGPR) can work directly by improving nutrient intake from the environment or indirectly by lowering plant diseases. Moreover, PGPR can defend plants from the harmful effects of environmental stresses such as drought, salinity, flooding, heavy metals, and phytopathogens (Mayak et al., 2004; Yildirim et al., 2006). The impact of PGPR on the growth of lettuce, bean, pepper, and canola under salinity stress was checked, and the detrimental effects of salinity were mitigated (Glick et al., 1997).

Inoculation of plants with 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase containing PGPR produced increased chlorophyll in maize and lettuce (Glick et al., 1997; Han and Lee, 2005). Numerous PGPRs have the ability to solubilize phosphorus and produce siderophores, enabling host plants to effectively absorb P and Fe-derived nutrients from the soil (Dodd and Perez-Alfocea, 2012). One of the most prevalent taxa of isolated endophytes among PGPRs is *Bacillus*, which offers a sustainable and environmentally acceptable method of plant growth promotion (PGP) through a several processes, including hormone synthesis, nutrient solubilization, and plant bio-protection (Shafi et al., 2017). Plant growth promoting bacteria that produce IAA, such as *Bacillus* species OSU-142, *Paenibacillus polymyxa* RC05, *Pseudomonas putida* RC06, and *Rhodobacter capsulatus* RC04, benefit plant growth development and nutrition uptake (Cakmakci et al., 2007). MDA was harmfully affected by salinity stress and significantly increased by 234.6% as compared to the non-saline control in *Acacia gerrardii* Benth. But inoculation with *Bacillus subtilis* (BERA 71) reduced the harmful effects of salinity on MDA (Hashem et al., 2016). Additionally, it has been observed that rhizobacteria regulate plant antioxidant enzymes including SOD, CAT, and APX to promote salt tolerance in plants (Yilmaz and Kulaz, 2019).

After potatoes, the tomato (*Lycopersicon esculentum* Mill.) is the second-most significant vegetable crop in the world. Due to their well-balanced composition of vitamins (A, B1, B2, B6, C, E, K, biotin, folic acid, nicotinic acid, and pantothenic acid), minerals (potassium, calcium, phosphorus, iron, and zinc), and antioxidants (carotenoids and polyphenolic compounds), tomatoes have an outstanding nutritional profile (Sharma et al., 2009). By altering the activity of crucial enzymes and the amounts of gibberellin (GA), salinity stress slows down and lowers the rate of germination of tomato seeds, respectively (Singh et al., 2012; Tanveer et al., 2020). Salinity stress has three-fold impacts, this results in ion imbalance, toxicity, and a reduction in water potential (La Pena and Hughes, 2007). A PGPR strain of *Achromobacter piechaudii* ARV8 has been shown to reduce salt stress in tomato plants (Mayak et al., 2004).

According to the Food and Agriculture Organization of the United Nations, soil salinity had an impact on more than 833 million hectares of land in 2021. It is estimated that more than 10% of agricultural land is affected by salinity, which poses a significant threat to global food security. Many countries in the Pacific, North Africa, South America, and the Middle East are the worst-affected regions (FAO, 2021). Approximately 2.1% of India's land surface, or 6.727 million hectares, is impacted by salt, of which 2.956 million hectares are salty and the remaining 3.771 million hectares are sodic (Arora and Sharma, 2017). About 75% of the soils in the country are affected by salt, and the largest states with salt-affected soils are Gujarat (2.23 million hectares), Uttar Pradesh (1.37 million

hectares), Maharashtra (0.61 million hectares), West Bengal (0.44 million hectares), and Rajasthan (0.50 million hectares) (Mandal et al., 2018). Nowadays, because of faulty and extensive irrigation practices, much of Gujarat's agricultural land and coastal land have become saline. The hasty application of various pesticides and agrochemicals has exacerbated the situation.

The present study was carried out to discover the behavior of the selected PGPR strains under salinity stress conditions as well as their role in tomato growth enhancement. Thus, the present study was undertaken. (a) Isolate bacteria with plant growth-promoting traits, (b) Identify and characterize the isolated bacteria based on biochemical characteristics, as well as confirm the bacterial genera through 16S rRNA sequence analysis, and (c) Understand the physiological and biochemical changes that occur after PGPR inoculation in the plant's rhizosphere during salt stress alleviation.

2 Materials and methods

2.1 Isolation and identification of microorganisms

2.1.1 Soil sample collection

Three plants were chosen for the isolation of bacteria from the rhizosphere of 'Kesudo' (*Butea monosperma* Lam), 'Kawaria' (*Cassia tora* L.), and 'Arjun' (*Terminalia arjuna* Roxb.) from the Little Rann of the Kutch in Gujarat (23°58′30″N, 70°12′19″E). By uprooting the root system, rhizosphere soil samples were carefully taken at a depth of 10 cm. They were then placed in a cold box for transportation to the laboratory, where they were kept at 4°C.

2.1.2 Bacterial isolation

In 50 ml Erlenmeyer flasks, 2 gm of soil were suspended in 20 ml of sterile distilled water to create soil suspensions. Erlenmeyer flasks were incubated for an hour at 150 rpm in an orbital shaker. 1 ml of the soil suspension and 9 ml of sterile distilled water were combined in glass tubes. By adding 1 ml of the suspension to 9 ml of sterile distilled water at 10^{-1} to 10^{-6} dilutions, serial dilutions were made. Inoculation of plates was done using these dilutions. 200 µl aliquots from various dilutions were transferred and spread on 5% NaCL supplemented nutrient agar plates, luria agar plates, minimal agar plates, and tryptone soya agar plates. All of these plates were incubated in an incubator at 37°C for 24 hours, and morphologically unique colonies that developed on the medium were separated and subcultured for future investigations. A total of 107 isolates were obtained, and slants were used to prepare and maintain the pure cultures of these isolates in their respective media. These 107 bacterial isolates were kept in the refrigerator at 4°C for future use.

2.1.3 Gram's staining

On a clean glass slide, smears of each bacterial isolate were made separately, dried, and then heat-fixed. The smear was treated with one drop of crystal violet solution and left to react for 45 seconds. The additional stain was removed using sterile water. One drop of Gram's iodine solution was then applied for 45 seconds. It was then rinsed with water before being submerged for one minute in 100 ml of absolute alcohol. After that, a drop of the counter stain safranin was put to the smear and left to react for one minute. It was gently cleaned with sterilized water, dried by air, placed in glycerin, and examined under oil immersion.

2.1.4 Plant growth promoting traits

2.1.4.1 IAA production

Bacterial isolates were grown for 48 hours at 30°C in nutrient broth containing 500 g/ml DL-tryptophan. Centrifuging was done on fully developed cultures at 4°C for 10 minutes at 10,000 rpm. IAA estimation was done using the supernatant. After that, the orthophosphoric acid (2 drops) and Salkowski reagent (4 ml) were mixed with the supernatant (2 ml). Pink colour manifestation implies IAA production. At 560 nm, the optical density was determined (Bric et al., 1991).

2.1.4.2 Determination of phosphate solubilization

Isolates of bacteria were grown in 50 ml of Pikovskaya's broth containing 100 mg of tricalcium phosphate, and the amount of soluble phosphorus released on the seventh day after inoculation was calculated. In order to calculate the amount of soluble phosphorous, the culture media was centrifuged for 10 minutes at 10,000 rpm (Olsen, 1954).

2.1.4.3 Estimation of siderophore production

To produce siderophore in 1 litre of distilled water, a small modification was made to the succinate (iron-free) medium. 0.5 ml of the old culture of each test isolate was added to 100 ml of medium in flasks and incubated at 30°C for 72 hours on a rotary shaker. Cell-free supernatant was obtained after centrifugation at 10,000 rpm for 20 minutes at 4°C. The supernatant was used for the estimation of siderophore.

To measure siderophores, the CAS (Chrome azurol S) liquid assay method was employed (Schwyn and Neilands, 1987). The CAS assay solution was combined with 0.5 ml of 72-hour-old cell-free supernatant, and 10 µl of shuttle solution was then added. After 10 minutes at room temperature, the colour intensity of the solution was measured with a UV-VIS spectrophotometer at 630 nm against a reference. A decrease in blue colour as expressed in percent siderophore units (% SU) was seen as a result of siderophore synthesis.

2.1.4.4 ACC deaminase activity

A modified version of Honma and Shimomura (1978) method, which measures the amount of α-ketobutyrate released during ACC hydrolysis, was used to quantify the activity of ACC deaminase. The amount of µmol of α-ketobutyrate generated by this reaction was determined by comparing the sample's absorbance at 540 nm to a standard curve of α-ketobutyrate with concentrations between 0.1 and 1 µmol.

2.1.5 Molecular identification and phylogenetic tree generation of bacterial isolates

The most efficient bacterial isolates were identified molecularly by sequencing their 16S rRNA gene. Out of 107 bacterial strains,

five were chosen for 16S rRNA analysis based on PGPR traits. Bacterial isolates' 16S rRNA sequences have been deposited into the GenBank database. The BLAST search tool was used to look for nucleotide sequence homology in the bacteria's 16S region. To align highly homologous sequences and generate a neighbour-joining tree, ClustalW and MEGA version 11.0 were used.

2.2 The effect of PGPR inoculation on tomato physiological and biochemical parameters under saline conditions

2.2.1 Preparation of the PGPR strain and inoculum

In this study, five different PGPR strains were used: *Bacillus pumilus* NCT4, *Bacillus firmus* NCT1, *Bacillus licheniformis* LCT4, *Bacillus cereus* LAT3, and *Bacillus safensis* LBM4. The PGPR strain's active cultures were prepared using nutrient broth and luria broth.

2.2.2 Plant material and study area

Seeds of tomato S-22 (*Lycopersicon esculentum* Mill.) (physical purity: minimum 98%, inert matter: maximum 2%, moisture: maximum 6%, pure seed: minimum 98%, and germination: minimum 70%) were collected on January 23, 2022, from the R.K. seed farms (Regd.), Delhi. The entire experiment was carried out in a greenhouse at Hemchandracharya North Gujarat University's botanical garden in Patan (23°51' N Latitude, 72°07' E Longitude) in Gujarat. For seedling emergence and growth, a nearby agriculture field's top 15 cm of surface soil (vertisol), which predominates in Gujarat's northern region, was obtained.

2.2.3 Salinization of soil

The collected surface soil was autoclaved, allowed to air-dry, and then passed through a 2 mm mesh. The 3 kg of soil was then thoroughly mixed with 7.8 g of sodium chloride (NaCl), resulting in an interstitial soil water salinity of 4 dsm^{-1}. To measure soil salinity, a soil suspension in distilled water with a soil:water ratio of 1:2 was prepared (Patel et al., 2009). After a thorough shaking, the soil suspension was left to stand for the night. Then, a conductivity metre (Systronic; Model 307) was used to measure the soil suspension's conductivity.

2.2.4 Experimental design

A total of 3 kg of soil was filled in 10 polythene bags (20.5 cm wide and 41 cm long) for each of the twelve treatments. After that, 120 bags were placed in an uncontrolled greenhouse with natural light and temperature. Healthy tomato seeds were surface sterilized for 1 minute with 0.1% Mercuric chloride ($HgCl_{2)}$ and rinsed six times with sterile distilled water before Biopriming in the appropriate active bacterial culture for 30 minutes. Seeds for control plants were soaked in sterile water for the same amount of time. The seeds were then dried in the shade for 30 minutes. On January 24, 2022, twenty seeds were gently pressed to a depth of approximately 10-15 mm in each bag after drying in the shade. Then, except for the control treatment, 30 ml of active culture was poured to each bag. On alternate days, 100 ml of tap water was given to moisten the soil's surface. The experiment was repeated three times in a completely randomized block design with ten replicates.

2.2.5 Seedling growth

Two of the seedlings that established first were kept in each bag, while others were uprooted as they appeared. After two months, the experiment was stopped. For each treatment, 20 plants were grown, and afterward they were cleaned with tap water to get rid of any soil that had stuck to the roots. Each seedling's morphological characteristics were noted. Up to 30 days following seeding, seed germination (%) was recorded. Using a scale, the height of the plant shoots was measured from the plant's tip to the stem's end using a scale, the length of the plant's roots was calculated from the collar region to the end of the root. A weighing machine was used to measure the fresh weight of the shoots and roots right after harvest. Before weighing the fresh weight of the shoot and root, extra moisture on them was blotted using tissue paper. After 5 days of drying in a hot air oven at 40°C, when a constant weight was attained, the dry weight of the shoot and root was measured using a weighing machine. On graph paper, the leaf area was marked out.

2.2.6 Biochemical parameters

2.2.6.1 Organic solutes (soluble sugars and proline)

The total soluble sugar concentration was estimated using the phenol-sulfuric acid method (Krishnaveni et al., 1984). 100 mg of leaves were hydrolyzed with 5 ml of 2.5 N HCl in a boiling water bath for 3 hours, and the reaction was then neutralized with solid sodium carbonate until the effervescence subsided. Then, the volume was raised to 100 ml, and it was centrifuged. Following that, 0.1 and 0.2 ml supernatant aliquots were obtained and increased to 1 ml in separate test tubes. The next step was to add 1 ml of phenol solution and 5 ml of 96% sulfuric acid to each test tube, shake them vigorously, and then place them in a water bath at 25 to 30°C for 20 minutes. At 490 nm, the chromophore was read. The total amount of carbohydrates was determined using the glucose standard curve.

The amount of proline in a sample was measured according to Patel et al. (2014). 500 mg of plant leaves were ground in 10 ml of 3% sulfosalicylic acid, and the mixture was centrifuged at 10,000 g for 10 minutes to extract the proline. In a test tube, an aliquot of 2 ml of supernatant was placed, and an equivalent volume of freshly made ninhydrin solution was added. The tubes were incubated for 30 minutes at 90°C in a water bath. The reaction was stopped using an ice bath after incubation. Then, while stirring constantly for 15 minutes, the reaction mixture was extracted with 5 ml of toluene. The tubes were left in the dark for 20 minutes in order to separate the supernatant of the toluene and aqueous phases. The toluene phase was carefully collected into a test tube, and the absorbance was then recorded at 520 nm. The concentration of proline was determined from a standard curve using the equation (µg proline in extract/111.5)/g of sample = µmol g^{-1} of fresh tissue.

2.2.6.2 Total chlorophyll content

The content of chlorophyll was determined according to Arnon (1949). Approximately 1 g of leaves were chopped into small pieces and homogenized with 80 percent (V/V) acetone in a precooled mortar and pestle. A small amount of $CaCO_3$ was added during the grinding. After centrifuging the extract for 15 minutes at 3000 rpm, it was diluted with 80 percent (V/V) acetone to make up to 25 ml. In a spectrophotometer, the OD of the clear solution was taken at 645 nm and 663 nm against a blank of 80% acetone. The following equation was used to determine the levels of chlorophyll 'a' and chlorophyll 'b':

Chlorophyll 'a' (µ/g/ml) = (12.7 × O.D. at 663 nm) – (2.69 × O.D. at 645 nm)

Chlorophyll 'b' (µ/g/ml) = (22.9 × O.D. at 645 nm) – (4.08 × O.D. at 663 nm)

Total chlorophyll (µ/g/ml) = (20.2 ×O.D. at 645 nm) + (8.02 × O.D. at 663 nm)

The content of chlorophyll was expressed as mg chlorophyll per gram fresh weight of the tissue.

2.2.6.3 Lipid peroxidation

Lipid peroxidation was estimated as the amount of malondialdehyde (MDA) determined by the TBA reaction as described by Heath and Packer (1968) with some modifications. 2 ml of 1% TCA were used to homogenize 200 mg of plant leaves before they were centrifuged at 10,000 × g for 15 minutes. 1 ml of the supernatant aliquot, 2 ml of 20% w/v TCA, and 2 ml of 0.5% TBA were mixed, and incubated at 95°C for 30 minutes, followed by a quick transfer to an ice bath in order to stop the reaction. The absorbance at 532 nm was measured following centrifugation at 10,000 × g for 5 min. The value at 532 nm was deducted from the value of non-specific absorbance at 600 nm. MDA concentration was determined from the extinction coefficient at 155 $mM^{-1}cm^{-1}$and it was defined as µmol g^{-1} fresh weight MDA.

2.2.6.4 Antioxidant enzymes

To prepare plant leaf extractions for analysis, 200 mg of plant material was homogenized in 2 ml of 0.2 M potassium phosphate buffer (pH 7.8 with 0.1 mM EDTA). At 4°C, the homogenate was centrifuged for 20 minutes at 15,000 × g. After that, the tissue extract was kept at -20°Cand used within 48 hours to estimate different antioxidant enzymatic activities.

A modified NBT (nitro blue tetrazolium) method developed by Beyer and Fridovich (1987) was used to measure the activity of superoxide dismutase (SOD). A 2 ml portion of the assay reaction mixture, which includes 50 mM phosphate buffer (pH 7.8), 2 mM EDTA, 9.9 mM L-methionine, 55 mM NBT, and 0.025% Triton X-100, was put into a test tube. After that, 40 µl of the diluted (×2) sample and 20 µl of 1 mM riboflavin were added, and then the reaction was started by illuminating the sample under a 15-W fluorescent tube. For the 10minute exposure, the test tubes were placed in a box that was lined with aluminum foil and placed about 12 cm away from the light source. The same reaction mixture was also contained in duplicate tubes, which were kept in the dark and utilized as blanks. The amount of enzyme per milligram of protein sample that causes 50% inhibition of the rate of NBT reduction at 560 nm was defined as one unit of SOD.

The activity of catalase (CAT) was measured according to Aebi (1984). A 3 ml assay mixture contained 10 mM H_2O_2 and 2 ml of plant leaf extract that had been 200 times diluted in a 50 mM potassium phosphate buffer with a pH of 7.0. The reduction in absorbance at 240 nm signaled the breakdown of hydrogen peroxide. The enzyme activity was calculated using the extinction coefficient of H_2O_2 (40 mM^{-1} cm^{-1} at 240 nm).

The activity of ascorbate peroxidase (APX) was determined using the method of Nakano and Asada (1981). 0.5 mM H_2O_2, 50 mM potassium phosphate buffer (pH 7.0), and 10 µl of plant leaf extract were all present in one ml of the assay mixture. To start the reaction, H_2O_2 was added last, and the drop in absorbance was measured for 3 minutes. The reduction in absorbance at 290 nm brought on by the oxidation of ascorbate during the reaction was used to determine the ascorbate peroxidase activity. The enzyme activity of APX was calculated using a reduced ascorbate extinction coefficient of 2.8 mM^{-1} cm^{-1}.

According to Smith et al. (1988) glutathione reductase (GR) activity was determined. A 10 µl aliquot of leaves extract was used in the assay along with 0.1 mM NADPH, 0.75 mM DTNB (Ellman's reagent; 5,5'-dithiobis-(2-nitrobenzoic acid)), and 1 mM GSSG in a total of 1 ml of assay volume. To begin the reaction, GSSG was added last, and the absorbance increase was measured for 3 minutes. The increase in absorbance at 412 nm was detected as GSH reduced DTNB to TNB during the process measured. The activity of glutathione reductase was calculated using the extinction coefficient of TNB (14.15 mM^{-1} cm^{-1}).

2.3 Mineral analysis of plant materials

On the leaves, mineral analyses were performed. A mortar and pestle were used to grind the plant leaves. Plant samples were evaluated in three subsamples. After triacid digestion (HNO_3: H_2SO_4: $HclO_4$ in the ratio of 10: 1: 4), the concentrations of Na, K, Ca, Fe, and Mg were measured using atomic absorption spectroscopy. The Mohr method was used to determine chloride using $K_2Cr_2O_7$ as an indicator in a titration of Cl ions with $AgNO_3$ standard solution (Johnson and Ulrich, 1959; Kacar and Inal, 2008). The chlorostannous molybdophosphoric blue colour technique in sulfuric acid was used to estimate the phosphorus level (Piper, 1944).

2.4 Statistical analysis

Multivariate cluster analysis was performed to construct a dendrogram based on the similarity matrix of physico-chemical parameters using the paired group (UPGMA) method with arithmetic averages and Bray-Curtis similarity index. Non-metric multidimensional scaling was done to group the treated and non-treated plants on the basis of similarity in different physico-chemical parameters. To determine the significant relationships between the measured parameters, principal component analysis was applied to all of the parameters. To evaluate the relationships between various physico-chemical parameters, Pearson's correlation coefficient analyses were performed. To find

significant variation between means, comparison and similarity groups of all measured parameters were performed using two-way ANOVA. These analyses were performed carried out using PAST: Palaeontological Statistics software package version 4.05 (Hammer et al., 2001).

3 Results

3.1 Isolation and identification of bacterial strain

There were 107 different bacterial strains detected in the soil from the rhizospheres of the "Kesudo" (*Butea monosperma* Lam.), "Kawaria" (*Cassia tora* L.), and "Arjun" (*Terminalia arjuna* Roxb.). These bacteria were both gram positive and gram negative.

3.1.1 Plant growth promoting traits

The traits that promote plant growth were evaluated in all 107 strains and found in 5 highly potent PGPR strains (NCT4, NCT1, LCT4, LAT3, and LBM4). Indole-3-acetic acid (IAA) and siderophore are produced by all five strains. Except for the LBM4 strain, all four strains were able to solubilize phosphate, and except for LAT3 and LBM4 strains, all three strains produced ACC deaminase (Table 1).

3.1.2 Molecular identification and phylogenetic tree generation

Five of the best performing strains according to their PGPR traits were from the *Bacillus* group. The 16S rRNA sequence were submitted to NCBI for identification of strain using the BLAST database and it was found that, isolate coded as NCT4 showed 100% sequence similarities with *Bacillus pumilus*18B (MN750426), NCT1 showed 98.64% sequence similarities with *Bacillus firmus* BTNGPSA5 (MK958537), LCT4 showed 98.8% sequence similarities with *Bacillus licheniformis* IHB B 10241 (KR233755), LAT3 showed 100% similarities with *Bacillus cereus* BLCC1-0148 (OP881599), and LBM4 showed 99.78% similarities with *Bacillus safensis* KMF402 (MT642941). All sequences were submitted to GeneBank which are accessible through accession number KF853108, KF853131, KF853123, KF853105, and KJ883295 respectively. Evolutionary relationships between the isolates generated by the Mega (version 11) programme (Figure 1). Next to the branches is the percentage of replicate trees in which the related taxa clustered together in the bootstrap test (1000 repetitions).

3.2 Effect of PGPR on physiological parameters of plant under saline conditions

In this work, the impact of PGPR on tomato plant growth in normal and saline conditions was investigated. Each PGPR strain significantly enhanced the tomato plants' root length, shoot height, leaf area, and germination percentage. Under 4 dsm^{-1} saline stress conditions, treated plants with NCT4, NCT1, LCT4, LAT3, and LBM4 had considerably larger shoot heights than untreated plants. As well, under 4 dsm^{-1} saline stress conditions, treated plants with NCT4, NCT1, and LCT4 had considerably longer plant roots than untreated plants (Table 2).

Moreover, in non-saline environments, the shoot height of the plant was significantly greater in NCT4, NCT1, LCT4, LAT3, and LBM4 treated plants than in non-inoculated plants, and the root length was significantly greater in NCT4, NCT1, and LCT4 treated plants than in non-inoculated plant. However, when treated with various microorganisms, tomato plant leaf area was also affected in both growing conditions (saline 4 dsm^{-1} and non-saline 0 dsm^{-1}). Tomato leaf area increased significantly when treated with the NCT4 strain under saline conditions, just as it increased considerably when treated with the NCT4 and NCT1 strains under non-saline conditions. In both saline and non-saline conditions, the NCT4 and NCT1 strains greatly boosted the tomato plants' germination percentage (Table 2).

Each of the PGPR strains significantly increased the tomato plant's shoot fresh weight, shoot dry weight, root fresh weight, and root dry weight. Under 4 dsm^{-1} salt stress conditions, treated plants with NCT4, NCT1, LCT4, LAT3, and LBM4 had considerably higher shoot fresh weights than untreated plants. As well, the shoot dry weight of the plant was significantly greater in NCT4,

TABLE 1 Plant growth-promoting rhizobacteria (PGPR) traits of the selected isolates.

Isolates	IAA production (µg/ml)	Phosphate solubilization (mg of P released from 100 mg of tricalcium phosphate)	% Siderophore Units (SU)	ACC deaminase activity (nmoL α- ketobutyrate mg^{-1} h^{-1})	Gen Bank Accession No.
Bacillus pumilus NCT4	42.5	11.69	48.7	354.8	KF853108
Bacillus firmus NCT1	45.7	6.17	41.2	316.5	KF853131
Bacillus licheniformis LCT4	27.3	5.85	56.1	641.2	KF853123
Bacillus cereus LAT3	15.6	2.08	49.3	ND	KF853105
Bacillus safensis LBM4	5.3	ND	38.2	ND	KJ883295

ND, Not Detected.

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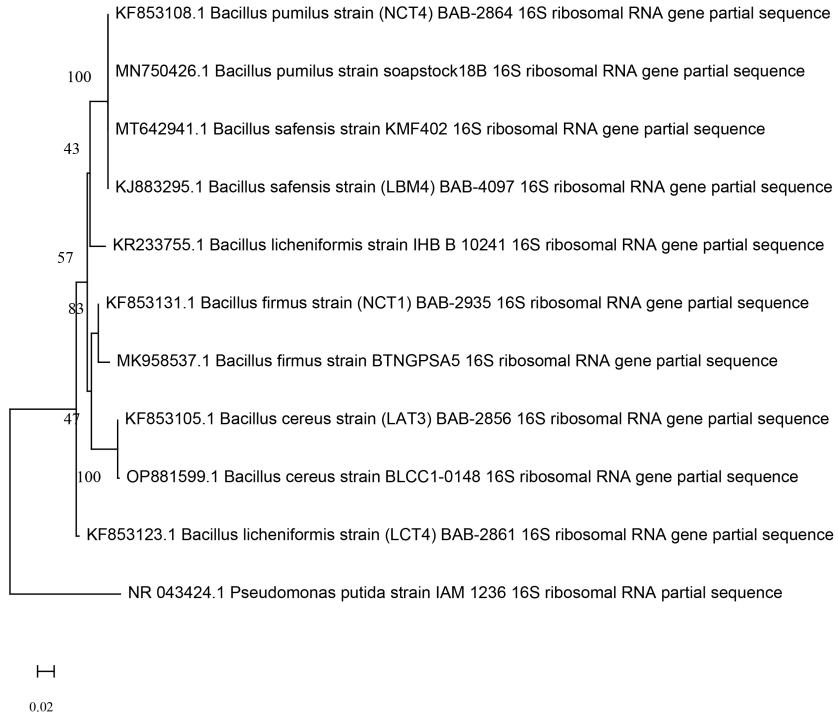

FIGURE 1
Phylogenetic relationship among the KF853108 *Bacillus pumilus* strain (NCT4), KJ883295 *Bacillus safensis* strain (LBM4), KF853123 *Bacillus licheniformis* strain (LCT4), KF853105 *Bacillus cereus* strain (LAT3), KF853131 *Bacillus firmus* strain (NCT1) and other type strains based on 16S rRNA gene sequences generated by neighbor end joining method.

NCT1, LCT4, LAT3, and LBM4 treated plants than in untreated plants under the4 dsm^{-1} saline stress condition. Additionally, in non-saline conditions, the NCT4-treated plant's shoot fresh weight was considerably higher than the untreated plant. The root fresh weight of the plant was significantly greater in NCT4 and NCT1-treated plants than in untreated plants under 4 dsm^{-1} saline stress conditions. Additionally, under 4 dsm^{-1} saline stress conditions, the root dry weight of the plant was considerably higher in NCT4 and

TABLE 2 Effects of plant growth-promoting rhizobacteria on the shoot height, root length, leaf area and germination % of tomato plants under saline conditions.

NaCl concentration	Treatment	Shoot height (cm)	Root length(cm)	Leaf Area (cm^2)	Germination (%)
0 dsm^{-1}	Non-inoculated	45.76 ± 2.4a	12.44 ± 1.7a	6.91 ± 0.5a	79 ± 6.4a
	NCT4	69.28 ± 4.8c	19.52 ± 1.8b	8.71 ± 0.9b	95 ± 5.4b
	NCT1	66.4 ± 3.4bc	17.76 ± 1.7b	8.29 ± 0.6b	93 ± 6.7b
	LCT4	60.32 ± 5.1bc	16.16 ± 2.1b	7.71 ± 0.7ab	86 ± 4.5ab
	LAT3	54.24 ± 5.3b	14.56 ± 1.0ab	7.32 ± 0.5ab	84 ± 3.8ab
	LBM4	51.71 ± 3.1b	14.24 ± 0.9ab	7.13 ± 0.6ab	83 ± 3.1ab
4 dsm^{-1}	Non-inoculated	37.9 ± 1.1a	10.68 ± 1.2a	5.72 ± 0.3a	63± 3.4a
	NCT4	57.35 ± 3.0c	16.15 ± 1.4c	7.18 ± 0.4b	76 ± 4.6b
	NCT1	54.45 ± 3.2bc	14.67 ± 1.2bc	6.85 ± 0.9ab	74 ± 4.3b
	LCT4	49.94 ± 4.2b	13.37 ± 1.3b	6.37 ± 0.4a	68 ± 3.3ab
	LAT3	45.49 ± 4.5b	12.5 ± 1.4ab	5.90 ± 0.2a	67 ± 3.2ab
	LBM4	42.75 ± 2.1b	11.77 ± 0.6ab	5.83 ± 0.4a	66 ± 2.6a

Data were analysed using the One-way ANOVA Tukey's multiple range test ($P<0.05$).
Different small letters have significant differences.

NCT1 treated plants than in untreated plants. The root fresh weight of the plant was significantly higher in the NCT4 treated plant than the untreated plant in the non-saline condition, and the root dry weight of the plant was significantly higher in the NCT4, NCT1, and LCT4 treated plant than the untreated plant (Table 3).

3.3 Effect of PGPR in a saline condition on proline and soluble sugar

Common organic solutes in higher plants like proline and soluble sugar build up as a result of stress. Proline and soluble sugar levels in PGPR-treated plants were considerably greater than those in untreated control plants, and inoculated plants accumulated more proline and soluble sugar under saline conditions than non-saline conditions. The soluble sugar content of salinity stressed tomato plants inoculated with three strains, NCT4, NCT1 and LCT4, was significantly increased, whereas in normal conditions, plants inoculated with strains NCT4, NCT1, LCT4, LAT3, and LBM4 were significantly increased. However, the proline content of salt stressed and non-salt stressed tomato plants inoculated with four strains, NCT4, NCT1, LCT4, and LAT3, was significantly increased (Figure 2).

3.4 Effect of PGPR on chlorophyll content and MDA under saline condition

In both normal and saline conditions, the chlorophyll content and MDA of tomato leaves were measured. Significant differences were found in the chlorophyll content of tomato plants as influenced by PGPR under saline and non-saline conditions. There was a significant raise in the chlorophyll of the leaves of tomato plants in NCT4, NCT1, LCT4, LAT3, and LBM4 treated plants compared to those of untreated plants under non-saline conditions. In contrast, there was a considerable increase in the chlorophyll of tomato plant leaves treated with NCT4, NCT1, LCT4, and LAT3 compared to untreated plants under 4 dsm^{-1} saline (Figure 2).

The results of lipid peroxidation of tomato plants as influenced by PGPR in saline and non-saline conditions revealed significant differences. Under non-saline conditions, lipid peroxidation of tomato plant leaves was significantly reduced in NCT4, NCT1, and LCT4 treated plants compared to untreated plants; however, there was no significant effect on lipid peroxidation of leaves in LAT3 and LBM4 treated plants. Under 4 dsm^{-1} saline conditions, lipid peroxidation of tomato plant leaves was significantly reduced in NCT4, NCT1, LCT4, and LAT3 treated plants compared to untreated plants; however, there was no considerable effect on lipid peroxidation of leaves in LBM4 treated plants (Figure 2).

3.5 Effect of PGPR on the activity of antioxidant enzymes in saline conditions

In the current work, the activity of four antioxidant enzymes (SOD, CAT, APX, and GR) was determined in leaf extracts from tomato plants grown under normal and saline conditions with and without PGPR inoculation. Inoculation along with all five PGPR strains boosted the activity of all four antioxidative enzymes under these conditions. To be more precise, tomato plants inoculated with NCT4 and NCT1 had considerably higher SOD enzyme activity under the non-saline condition compared to the control condition,

TABLE 3 Effects of plant growth-promoting rhizobacteria on the shoot fresh weight, shoot dry weight, root fresh weight, and root dry weight of tomato plants under saline conditions.

NaCl Concentration	Treatment	Shoot fresh weight (gm)	Shoot dry weight (gm)	Root fresh weight (gm)	Root dry weight (gm)
0 dsm^{-1}	Non-inoculated	9.58 ± 0.8a	3.44 ± 0.4a	0.59 ± 0.07a	0.21 ± 0.02a
	NCT4	11.13 ± 1.1b	3.99 ± 0.4a	0.91 ± 0.04b	0.33 ± 0.03c
	NCT1	10.81 ± 0.9ab	3.87 ± 0.4a	0.79 ± 0.08ab	0.28 ± 0.02bc
	LCT4	10.54 ± 0.9ab	3.77 ± 0.4a	0.71 ± 0.11a	0.25 ± 0.01b
	LAT3	10.21 ± 1.1a	3.64 ± 0.4a	0.66 ± 0.08a	0.23 ± 0.03ab
	LBM4	10.18 ± 0.8a	3.62 ± 0.2a	0.64 ± 0.10a	0.22 ± 0.01a
4 dsm^{-1}	Non-inoculated	5.74 ± 0.5a	2.05 ± 0.1a	0.49 ± 0.03a	0.16 ± 0.01a
	NCT4	9.35 ± 0.8b	3.35 ± 0.2bc	0.74± 0.07b	0.26 ± 0.01c
	NCT1	9.03 ± 0.4b	3.23 ± 0.1bc	0.63± 0.07b	0.22 ± 0.01b
	LCT4	8.85 ± 0.6b	3.17 ± 0.2bc	0.57± 0.08a	0.19 ± 0.02ab
	LAT3	8.56 ± 0.8b	3.06 ± 0.2b	0.53± 0.04a	0.18 ± 0.02a
	LBM4	8.42 ± 0.4b	3.0 ± 0.1b	0.52± 0.05a	0.18 ± 0.03a

Data were analysed using the One-way ANOVA Tukey's multiple range test ($P<0.05$).
Different small letters have significant differences.

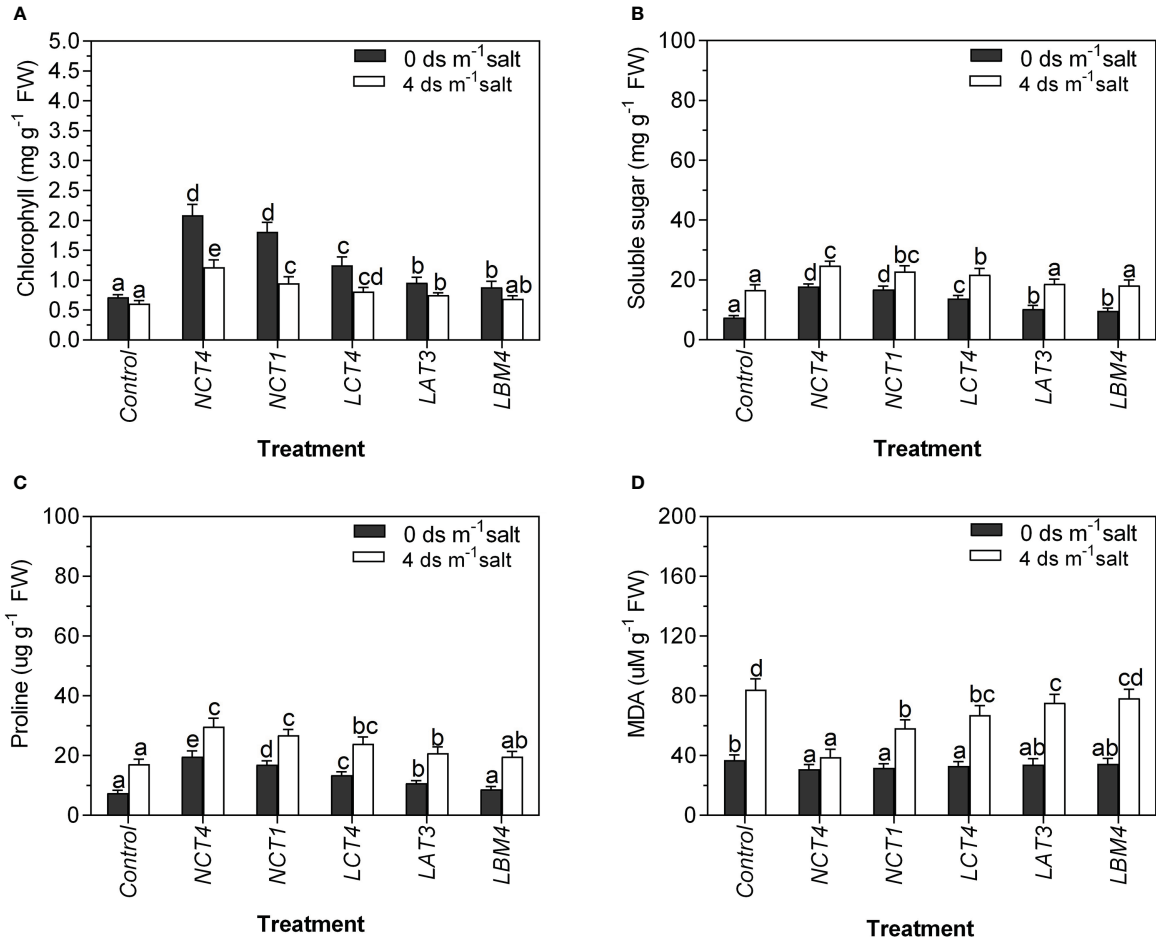

FIGURE 2
Effects of plant growth promoting rhizobacteria (PGPR) on the **(A)** Chlorophyll **(B)** Soluble sugar **(C)** Proline **(D)** MDA leaf extracts of tomato plant under saline condition. Data were analysed using the One-way ANOVA Tukey's multiple range test ($P<0.05$). Different small letters have significant differences.

and there was no significant effect on LCT4, LAT3, and LBM4 under the non-saline condition. Under 4 dsm^{-1}saline condition, there was a considerable increase in SOD of tomato plant leaves in NCT4, NCT1, and LCT4 treated plants compared to untreated plants, but there was no significant effect on SOD of leaves in LAT3 and LBM4 treated plants (Figure 3).

Both in saline and non-saline conditions, tomato plants treated with all five strains showed considerably higher catalase enzyme activity. Furthermore, in non-saline conditions, tomato plants inoculated with NCT4, NCT1, LCT4, and LAT3 had much higher APX enzyme activity than control plants, but LBM4 treatment had no discernible impact on leaves' APX activity. Whereas, under 4 dsm^{-1} saline conditions, compared to untreated plants, all five PGPR-treated tomato plants showed a considerable rise in APX of their leaves (Figure 3).

The GR enzyme activity in tomato plants treated with NCT4 was significantly increased in saline and non-saline conditions compared to the control, but there was no significant effect on the GR enzyme activity of leaves in NCT1, LCT4, LAT3, and LBM4 treated plants in non-saline and saline conditions. These results demonstrate that PGPR treated plants' elevated antioxidant enzyme activity may contribute in their higher salt tolerance (Figure 3).

3.6 Effect of PGPR on mineral analysis of plant materials under saline condition

The effects of PGPR on Na content in tomato plants in saline and non-saline conditions revealed significant differences. There was a considerable decrease in Na content of leaves of tomato plants in NCT4 treated plants compared with untreated plants under non-saline conditions, and there was no significant effect on the Na content of leaves in NCT1, LCT4, LAT3, and LBM4 treated plants under non-saline conditions. Under 4 dsm^{-1} saline conditions, there was a significant decrease in Na content of tomato plant leaves in all five PGPR (NCT4, NCT1, LCT4, LAT3, and LBM4) treated plants compared to untreated plants (Figure 4).

Significant differences were noticed in tomato plants with respect to Cl contents influenced by PGPR in saline and non-saline conditions. Under non-saline conditions, the Cl content of tomato leaves was significantly lower in NCT4, NCT1, and LCT4treated plants than in untreated plants, whereas there was no notable effect on the Cl content of leaves in LCT4, LAT3, and LBM4treated plants. Salinity stress conditions of 4 dsm^{-1}, the Cl of tomato plant leaves was significantly lower in the NCT4-treated plant than in the untreated plant, but there was no considerable

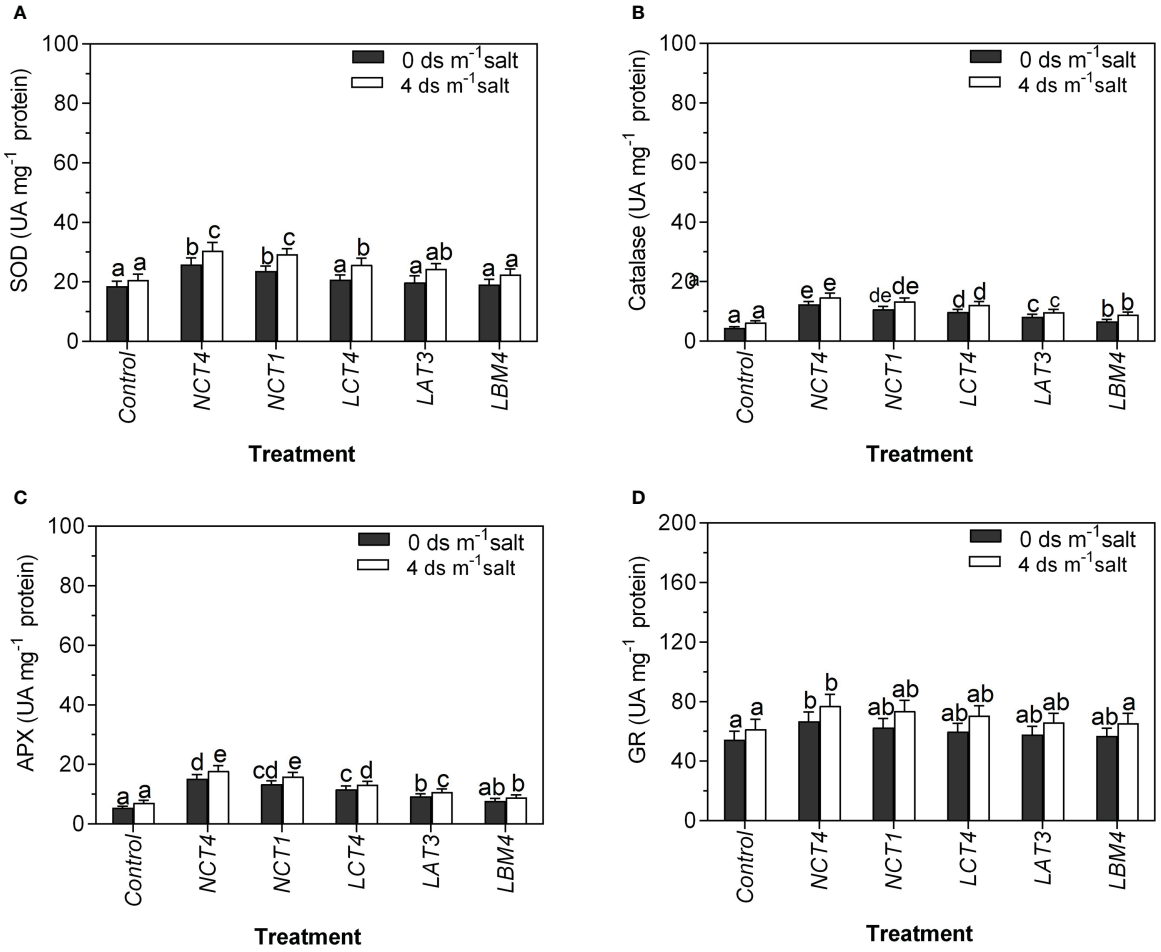

FIGURE 3
Effects of plant growth promoting rhizobacteria (PGPR) on the antioxidant enzyme activity of leaf extracts of tomato plant under saline condition. **(A)** Superoxide dismutase (SOD) activity **(B)** Catalase activity **(C)** Ascorbate peroxidase (APX) activity **(D)** Glutathione reductase (GR) activity. Data were analysed using the One-way ANOVA Tukey's multiple range test (P<0.05). Different small letters have significant differences.

effect on the Cl content of leaves in the NCT1, LCT4, LAT3, and LBM4 treated plants (Figure 4).

The results of Mg content of tomato plants as influenced by PGPR under saline and non-saline conditions revealed significant differences. Both in non-saline and saline environments, there was a major increase in Mg content of leaves of tomato plant in all five PGPR (NCT4, NCT1, LCT4, LAT3, and LBM4) treated plants compared to untreated plants (Figure 4).

Significant differences were noticed in tomato plants with respect to Ca content as influenced by PGPR under saline and non-saline conditions. Under non-saline conditions, the Ca content of tomato plant leaves was notably higher in NCT4, NCT1, and LCT4 treated plants than in untreated plants, with no significant effect on the Ca content of leaves in LAT3 and LBM4 treated plants. Under 4 dsm^{-1} saline stress conditions, the Ca content of tomato leaves was significantly higher in NCT4 and NCT1 treated plants than in untreated plants, but there was no considerable effect on the Ca content of leaves in LCT4, LAT3, and LBM4 treated plants (Figure 4).

The results for K content of tomato plants as influenced by PGPR under saline and non-saline conditions revealed significant differences. Under non-saline circumstances, there was a significant increase in K of tomato leaves in NCT4, NCT1, and LCT4 treated plants compared to untreated plants, but there was no significant effect on K content of leaves in LAT3 and LBM4 treated plants. Under 4 dsm^{-1}saline conditions, there was a significant raise in K of tomato leaves in NCT4, NCT1, LCT4, and LAT3 treated plants compared to untreated plants, but there was no significant effect on K of leaves in LBM4 treated plants (Figure 4).

Significant differences were noticed in tomato plants with respect to P contents influenced by PGPR both in salinized and non-salinized environments. The P content of leaves of the tomato plant was significantly higher in all five PGPR (NCT4, NCT1, LCT4, LAT3, and LBM4) treated plants than in untreated plants under non-saline and 4 dsm^{-1} saline stress conditions (Figure 4).

Significant differences were noticed in tomato plants with respect to Fe contents influenced by PGPR under saline and non-saline conditions. Under conditions that are not salty, the Fe content of tomato plant leaves was notably higher in NCT4 treated plants than in untreated plants, with no significant impact on the Fe content of leaves in NCT1, LCT4, LAT3, and LBM4 treated plants. Under 4 dsm^{-1} saline stress conditions, the Fe content of tomato plant leaves was significantly higher in NCT4

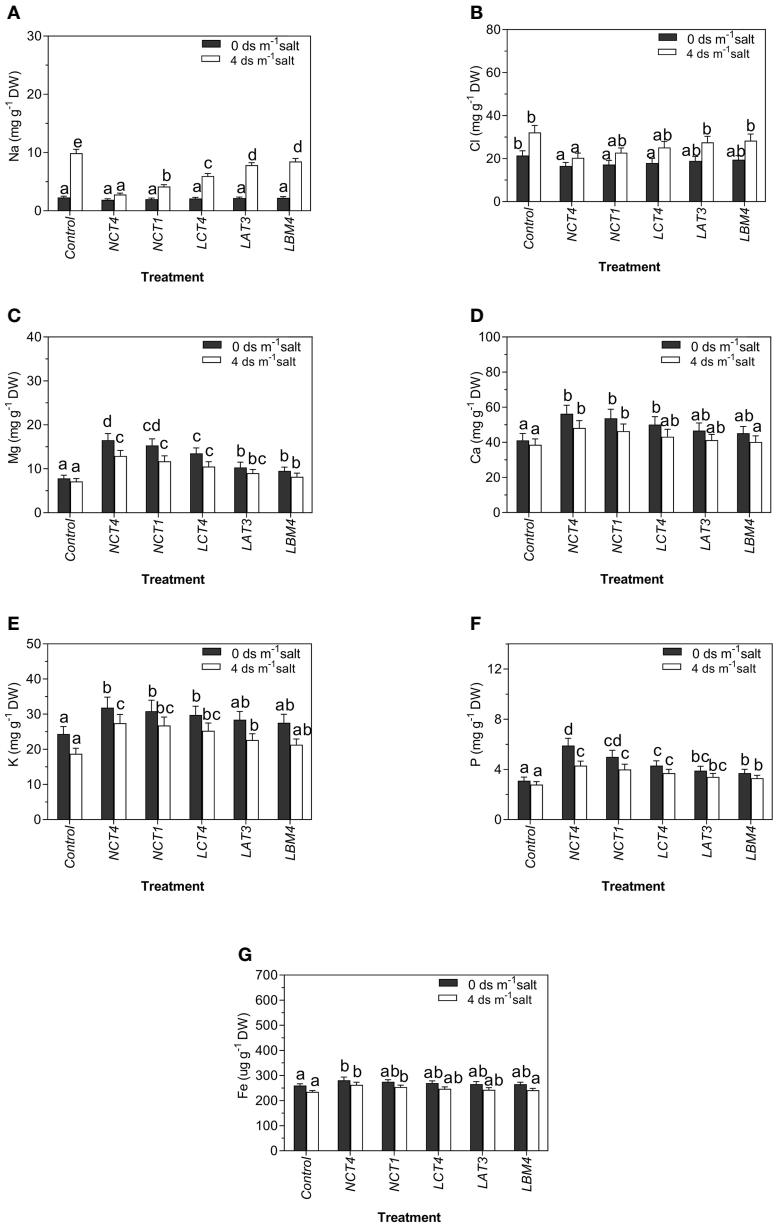

FIGURE 4
Effects of plant growth promoting rhizobacteria (PGPR) on the **(A)** Na **(B)** Cl **(C)** Mg **(D)** Ca **(E)** K **(F)** P **(G)** Fe of leaf extracts of tomato plant under saline condition. Data were analysed using the One-way ANOVA Tukey's multiple range test (P<0.05). Different small letters have significant differences.

and NCT1-treated plants than in untreated plants, but there was no considerable effect on the Fe content of leaves in LCT4, LAT3, and LBM4-treated plants (Figure 4).

3.7 Statistical analysis of plant growth and physico-chemical parameters

From the correlation analysis, it was observed that there was positive correlation of soluble sugar and proline content with SOD, catalase, APX and GR which suggests that these parameters help in increasing the activity of these enzymes during stress. There was positive correlation of germination rate with Fe and K content whereas negative correlation of germination rate with MDA and Cl content which suggests that Fe and K promoted germination of plants during stress whereas MDA and Cl inhibited germination of plants during stress. There was negative correlation of Fe and K with MDA, Na and Cl content which suggests that they reduce the effect of these oxidative parameters in treated plants. There was negative correlation of shoot length, root length and leaf area with the chlorine content which inhibited the growth of plants during stress. However, there was positive correlation of shoot length, root length and leaf area with the chlorophyll, Mg, Ca, P, Fe and K content which suggested that there was remarkable growth in treated plants due to the increase in these (chlorophyll, Mg, Ca, P, Fe and K) physico-chemical parameters (Figure 5 and Table S1).

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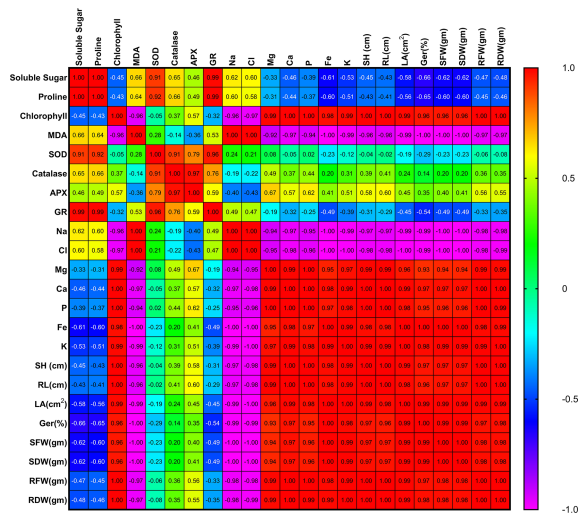

FIGURE 5
Correlation matrix among the different parameters of tomato plant influenced by salt stress as well as PGPR inoculation. Here, MDA, Malondialdehyde; SOD, Superoxide dismutase; APX, Ascorbate peroxidase; GR, Glutathione reductase; Na, Sodium; Cl, Chloride; Mg, Magnesium; Ca, Calcium; P, Phosphorus; Fe, Iron; K, Potassium; SH, Shoot height; RL, Root length; LA, Leaf area; Ger(%), Germination (%); SFW, Shoot fresh weight; SDW, Shoot dry weight; RFW, Root fresh weight; RDW, Root dry weight.

The Multivariate cluster analysis was done to detect the similarity between the treated and non-treated plants. Cluster analysis grouped the treated and non-treated plants into four groups on the basis of growth and physico-chemical characteristics. The group A included plants grown in presence of NCT4 and NCT1 culture, that had high germination rate, shoot length, root length, leaf and area. In physico-chemical parameters plants grown in presence of NCT4 and NCT1 had high chlorophyll, Mg, Ca, Fe, P and K content whereas low Na and Cl content. It had moderate MDA, proline and soluble sugar content and moderate enzyme activity of superoxide dismutase (SOD), catalase, Ascorbate peroxidase (APX) and Glutathione reductase (GR) in these plants. Group B included control and plants grown in presence of LCT4, LAT3, and LBM4 culture, that had high germination rate, shoot length, root length, and leaf area which was similar to control plants. In physico-chemical parameters, it had moderate chlorophyll, Mg, Ca, Fe, P, K, MDA, proline and soluble sugar content whereas low Na and Cl content. There was remarkable low enzyme activity of superoxide dismutase, catalase, Ascorbate peroxidase and Glutathione reductase in these plants. Group C included plants grown in presence of NCT4+salt, NCT1+salt and LCT4+salt, that had moderate germination rate, shoot length, root length, and leaf area. In physico-chemical parameters, it had moderate Mg, Ca, Fe, P, K, Na, Cl and chlorophyll content whereas high MDA, proline and soluble sugar content. There was remarkable high enzyme activity of superoxide dismutase, catalase, Ascorbate peroxidase and Glutathione reductase in these plants. Group D included control plants grown in presence of salt and plant grown in presence of LAT3+salt and LBM4+salt, that had low germination rate, shoot length, root length, and leaf area. In physico-chemical parameters, it had low Mg, Ca, Fe, P, K content whereas high Na, and Cl content. It had moderate chlorophyll proline and soluble sugar content whereas high MDA, content. There was moderate enzyme activity of superoxide dismutase, catalase, Ascorbate peroxidase and Glutathione reductase in these plants as compared to other plants (Figure 6).

Similar clusters were also formed by non-metric multidimensional scaling (MDS) (Figure S1) which supported the results of multivariate cluster analysis. From the principal component analysis, it was observed that there were 99.99% total variations retained on the basis of the eigen value. The principal component analysis divided the data in to twelve groups on the basis of significant variation in growth and physico-chemical parameters. Group 1 included control plants that varied from treated plants at 98.85% whereas treated plants varied from each other in range from 0.9 to 0.00003% (Table S2). The heatmap of principle component analysis also had the similar clustering as observed in multivariate cluster analysis which supports the results (Figure 7).

4 Discussion

This work successfully established the salt-tolerance and plant growth-promoting abilities of particular PGPR isolates, as well as their impact on enhancing the salinity reduction of tomato S-22 in a greenhouse environment. One essential plant growth-promoting characteristic of PGPR is the synthesis of indole-3-acetic acid, a signal molecule in the control of plant development. In the current

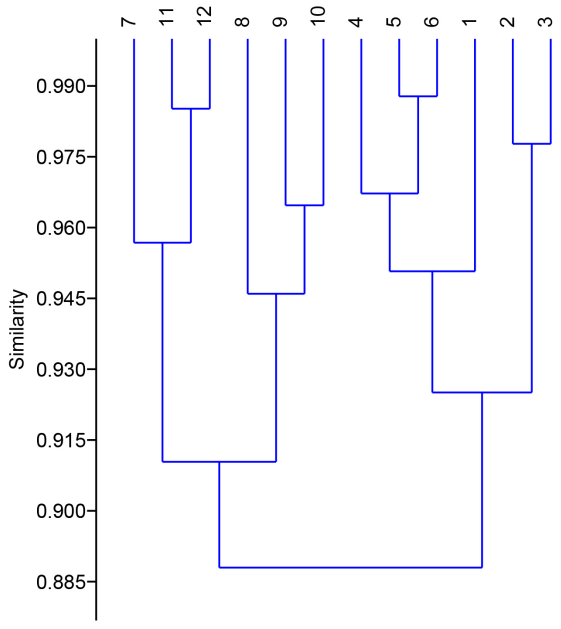

FIGURE 6
Dendrogram based on Paired group (UPGMA) algorithm using similarity index Bray-curtis for clustering of control and treated plants on the basis of growth and physico-chemical parameters. (1. Control plants 2. NCT4 treated plants 3. NCT1 treated plants 4. LCT4 treated plants 5. LAT3 treated plants 6. LBM4 treated plants 7. Control plants grown in presence of salt 8. NCT4+salt treated plants 9. NCT1+salt treated plants 10. LCT4+salt treated plants 11. LAT3+salt treated plants 12. LBM4+salt treated plants).

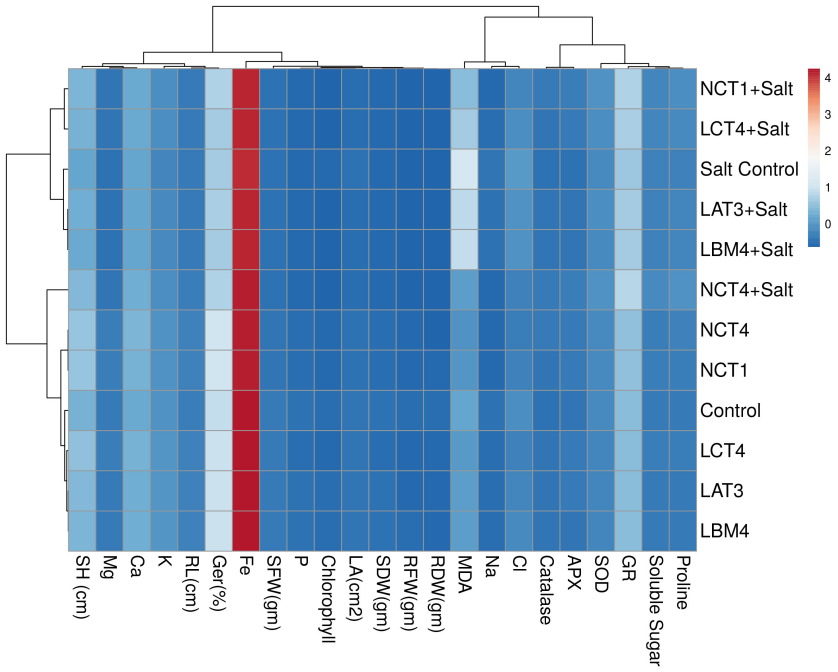

FIGURE 7
Heatmap represents grouping of control and treated plants in PCA on the basis of growth and physico-chemical parameters.

investigation, NCT1 produced the most IAA, NCT4 solubilized the most P, and LCT4 produced the most siderophore and ACC deaminase. Additionally, according to Aslam and Ali (2018), ACC-deaminase activity in halotolerant in saline conditions, the bacterial species *Arthrobacter, Brevibacterium, Bacillus, Gracilibacillus, Virgibacillus, Salinicoccus*, *Pseudomonas*, and *Exiguobacterium* encouraged the growth of maize. The remarkable capacity of *B. tequilensis* SSB07 to create biologically active metabolites including gibberellins, indole-3-acetic acid, and abscisic acid was previously demonstrated by Kang et al. (2019). Similar to this, a *Bacillus aryabhattai* (AB211) strain was identified by Bhattacharyya et al. (2017) that produces a clear zone on a Pikovskaya's agar plate, showing P solubilization. Chookietwattana and Maneewan (2012) previously found *Bacillus megaterium* A12 as a productive halotolerant P solubilizing bacterium in a saline environment. Patel et al. (2023) also found that endophytic *Bacillus safensis* (BS) and rhizospheric *Bacillus haynesii* (BH) strains were able to produce indole-3-acetic acid, gibberellic acid, hydrogen cyanide, ammonia, exopolysaccharides, protease, chitinase, amylase, cellulase, 1-amino cyclopropane-1-carboxylic acid deaminase, and solubilized minerals such as phosphorous, zinc, and potassium. Dutta et al. (2023) reported that *Bacillus* sp. strain PnD, which was isolated from the Indian Sundarban Mangrove Forest, conferred plant growth promoting (PGP) traits like indole 3-acetic acid (IAA) production, phosphate solubilization, and siderophore production.

Additionally, prior studies have demonstrated that PGPR causes the release of metal chelating compounds into the rhizosphere, such as iron chelating siderophores, and that bacteria that produce siderophores have an impact on the uptake of several metals by plants, including Fe, Zn, and Cu. The five PGPR strains utilized in this work can all create siderophore, which is interesting because Dimkpa et al. (2008) discovered that PGPR and other microbes can affect plant stress tolerance by affecting the bioavailability of metal ions needed by their host plants. Lower ethylene levels brought on by the presence of the chosen PGPR containing ACC deaminase generating efficiency could be the cause of the root elongation. Longer roots may have formed as a result of these rhizobacterial strains' ability to lower endogenous ethylene levels through the activity of ACC deaminase. The additional benefit of this is an increase in shoot height. Under 6 dsm^{-1}NaCl stress in the presence of PGPR, Nadeem et al. (2006) noted a comparable elongation in the root length and shoot height of maize. In this investigation, the NCT4, NCT1, and LCT4 strains could produce ACC deaminase (Table 1).

Significant changes were found in plant physiological parameters of tomato plants that were affected by PGPR, including germination %, shoot height, root length, leaf area, shoot fresh weight, shoot dry weight, root fresh weight, and root dry weight (Tables 2, 3). According to Jha and Subramanian (2013), paddy rice (*Oryza sativa L.*) 'GJ-17' grown in a greenhouse showed 16% greater germination, 27% higher dry weight, and 31% higher plant height in PGPR infected plants under saline conditions. Sen and Chandrasekhar (2014) found that rice genotype ADT43 plants treated with *Pseudomonas* strain greatly enhanced plant height, root length, and dry weight of shoot and root even under salt stress, but plants planted without any treatment grew less. Pérez-Rodriguez et al. (2022) found that *Enterobacter* 64S1 and *Pseudomonas* 42P4 inoculation increased root and shoot dry weight, stem diameter, plant height, and leaf area of tomato compared to control noninoculated plants under saline stress conditions, reversing the effects of salinity. Fan et al. (2016) reported that seed germination,

seedling length, vigour index, and plant fresh and dry weight were all increased by inoculating salt-stressed plants with *Arthrobacter* and *Bacillus megaterium* strains. Masmoudi et al. (2021a) also found that *Bacillus velezensis* FMH2-treatment promoted tomato plant growth (root structure, plant elongation, leaf emission, fresh and dry weights, and water content) in absence as well as in presence of salt stress. According to Tank and Saraf (2010), pot experiments on tomato plants stressed with 2% NaCl showed that C4 and T15 were the most successful growth enhancers. When compared to NaCl added untreated seedlings as well as in the absence of NaCl, C4 demonstrated a 50% increase in root length and shoot height. Tomato plants treated with *Enterobacter hormaechei* (MF957335) in saline circumstances dramatically increased their fresh biomass, shoot length, and root length (Ranawat et al., 2021). According to Manh Tuong et al. (2022), under salt stress conditions, the fresh weight of *Stenotrophomonas* sp. SRS1-inoculated *Arabidopsis* and tomato plants was noticeably higher than that of non-inoculated plants. According to Aini et al. (2021), applying saline-tolerant bacteria four times significantly increased plant height (23.36%), leaf area (96.49%), dry weight of the plant (103.59%), and fresh weight of the fruit (85.51%) as compared to not applying bacteria.

In the current study, we found that the levels of both proline and soluble sugar were raised in the PGPR treated tomato plants under salty circumstances. Increased soluble sugar content is another important defense strategy for plants experiencing salt stress (Upadhyay et al., 2012) (Figure 2). Proline promotes osmotic adjustment at the cellular level, shielding intracellular macromolecules from dehydration, and it also serves as a hydroxyl radical scavenger, so plants acquire proline as an adaptive response to both general stress and salt. Therefore, through enhancing metabolic defense mechanisms, the PGPR strains most likely aid in promoting plant development under salinity stress (Weisany et al., 2012).

Han and Lee (2005) claim that PGPR, including *Serratia* and *Rhizobium* species, enhance lettuce growth, nitrogen uptake, and chlorophyll content at various soil salinity levels. The most recent research also demonstrates that PGPR inoculation boosted the chlorophyll content of tomato leaves (Figure 2). Similar results were obtained by Hahm et al. (2017), who discovered that all three PGPR strains (*Microbacterium oleivorans* KNUC7074, *Brevibacterium iodinum* KNUC7183, and *Rhizobium massiliae* KNUC7586) when inoculated into plants led to higher chlorophyll concentrations than plants that weren't inoculated.

El-Beltagi et al. (2022) discovered that cherry tomato plants treated with *Azospirillum* and *Azotobacter* had higher leaf chlorophyll content than untreated plants under saline conditions. Inoculating paddy plants with a single PGPR reduced lipid peroxidation by one time, whereas combining two PGPR lowered the level by 1.6 times, according to Jha and Subramanian (2014) research. In both saline and non-saline circumstances, the current study found that PGPR dramatically decreased lipid peroxidation in tomato plants (Figure 2). According to Hashem et al. (2016), MDA was negatively impacted by salinity and dramatically elevated by 234.6% in comparison to the *Acacia gerrardii* Benth-saline control. However, *Bacillus subtilis* (BERA 71) inoculation lessened the negative effects of salt on MDA.

Reactive oxygen species (ROS) that are produced as a result of salt stress can injure plants by producing oxidative stress. Superoxide dismutase (SOD), dehydroascorbate reductase (DHAR), catalase (CAT), glutathione reductase (GR), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX) are just a few of the antioxidant enzymes that are involved in antioxidant systems, which are essential for protecting both plants and animals from oxidative stress (Caverzan et al., 2012).

According to El-Esawi et al. (2019) research, plants inoculated with *Azospirillum lipoferum* FK1 shown greater antioxidant gene expression, which boosted the production of antioxidant enzymes and promoted chickpea growth and development. In plant systems, enzymes and redox metabolites cooperate to detoxify ROS. For instance, CAT catalyses the conversion of H_2O_2 to oxygen and water, while APX and GPX both catalyze the conversion of H_2O_2 to water. No matter the growing conditions (normal or saline), the antioxidant enzyme activities (SOD, CAT, APX, and GR) in leaf extracts of PGPR treated tomato plants were significantly higher than those seen in non-inoculated control plants (Figure 3). Our results corroborate those of Gururani et al. (2013), who discovered that PGPR-treated potato plants exposed to various stressors had increased activity of ROS-scavenging enzymes like APX, CAT, DHAR, GR, and SOD (salt, drought, and heavy metals). Khan et al. (2023) also found that antioxidative enzymes (SOD, CAT, APX and GR) increased by 58.40, 25.65, 81.081 and 55.914%, respectively, over salt-treated plants through the application of *Pseudomonas fluorescens*. Additionally, salt-stressed okra plants treated with the PGPR *Enterobacter* sp. UPMR18 showed enhanced SOD, APX, and CAT activities (Habib et al., 2016).

In the current study, it was discovered that the sodium and chloride contents of tomato plants as affected by PGPR in saline and non-saline circumstances were significantly lower (Figure 4). Mohamed and Gomaa (2012) found that salinity stress greatly raised the Na^+ and Cl^- concentration in the roots and leaves of radish plants. Inoculating radish seeds with *Pseudomonas fluorescens* and *Bacillus subtilis* greatly lowered the amounts of Na^+ and Cl^-. Additionally, Yildirim et al. (2008) found that radish plants inoculated with bacteria under saline stress have lower Na^+ and Cl^- contents than uninoculated plants.

Under both saline and non-saline environments, the study discovered a considerable rise in the magnesium, calcium, potassium, and phosphorus content of tomato plants as impacted by PGPR (Figure 4). These results concur with those of Mohamed and Gomaa (2012), who found that the Mg^{2+}, Ca^{2+}, K, and P contents of radish roots and leaves were decreased by saline stress. Radish seeds were inoculated with *Pseudomonas fluorescens* and *Bacillus subtilis*, which dramatically raised the amounts of Mg^{2+}, Ca^{2+}, K, and P. Masmoudi et al. (2021b) also reported that *Bacillus spizizenii* FMH45 inoculation significantly decreased endogenous Na^+ accumulation, increased K^+ and Ca^{2+} uptake of tomato plants exposed to salt stress. Karlidag et al. (2013) discovered that PGPR inoculation enhanced the Mg^{2+}, Ca^{2+}, K, and P content of strawberry leaf and root in comparison to non-inoculated plants in salinity conditions. Solórzano-Acosta et al. (2023) found that Rhizobacteria *Pseudomonas plecoglossicida*, and *Bacillus subtilis* contributed to the accumulation of potassium in the leaves, compared to the uninoculated control under salt stress condition.

In the current study, significant variations in tomato plants' iron content as impacted by PGPR under saline and non-saline conditions were found (Figure 4). According to Karlidag et al. (2013), in salinity conditions, PGPR inoculations increased the Fe content of strawberry leaf and root compared to non-inoculated plants. *Kocuria erythromyxa* EY43 had the greatest Fe content, followed by *Bacillus atrophaeus* EY6, *Staphylococcus kloosii* EY37, and *Bacillus spharicus* EY30. Ekinci et al. (2014) claimed that PGPR raised the Fe content of cauliflower.

5 Conclusion

According to the current study, *Bacillus* sp., a bacteria that promotes plant growth, can enhance salt stress-induced plant growth and development by solubilizing phosphate and generating ACC deaminase, siderophore, and IAA. Increased levels of ROS-scavenging enzymes (SOD, CAT, APX, and GR), as well as proline and soluble sugar accumulation, which serve as osmoregulants, were also linked to tomato seedlings' tolerance to salt stress. The goal of this study is to find effective strains that enhance tomato development in both non-stressed and salt-stressed environments. These strains were isolated from the rhizosphere of 'Kesudo', 'Kawaria', and 'Arjun' plants. Accordingly, the results of the present study imply that PGPR can reduce the harmful effects of salt stress on plants, presumably by serving as elicitors that boost plant tolerance to a variety of abiotic stresses.

6 Future research

Future research would focus on identifying gene expression and mRNA expression patterns associated with tomato tolerance mechanisms.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding authors.

Author contributions

Conceptualization and supervision: AsP, SB and MJ. Investigation and methodology: AnP, VY and DP. Original draft preparation and analysis: DA, HK and JT. Review and final editing: AsP, MJ, SB and JT. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1168155/full#supplementary-material

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Muhammad Musa Khan,
Zhejiang University, China

REVIEWED BY
Babar Iqbal,
Jiangsu University, China
Abhay K. Pandey,
North Bengal Regional R & D Center, India

*CORRESPONDENCE
Jiyun Nie
jiyunnie@163.com
Yinping Li
liyinpingxx@163.com

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Comprehensive review on patulin and *Alternaria* toxins in fruit and derived products

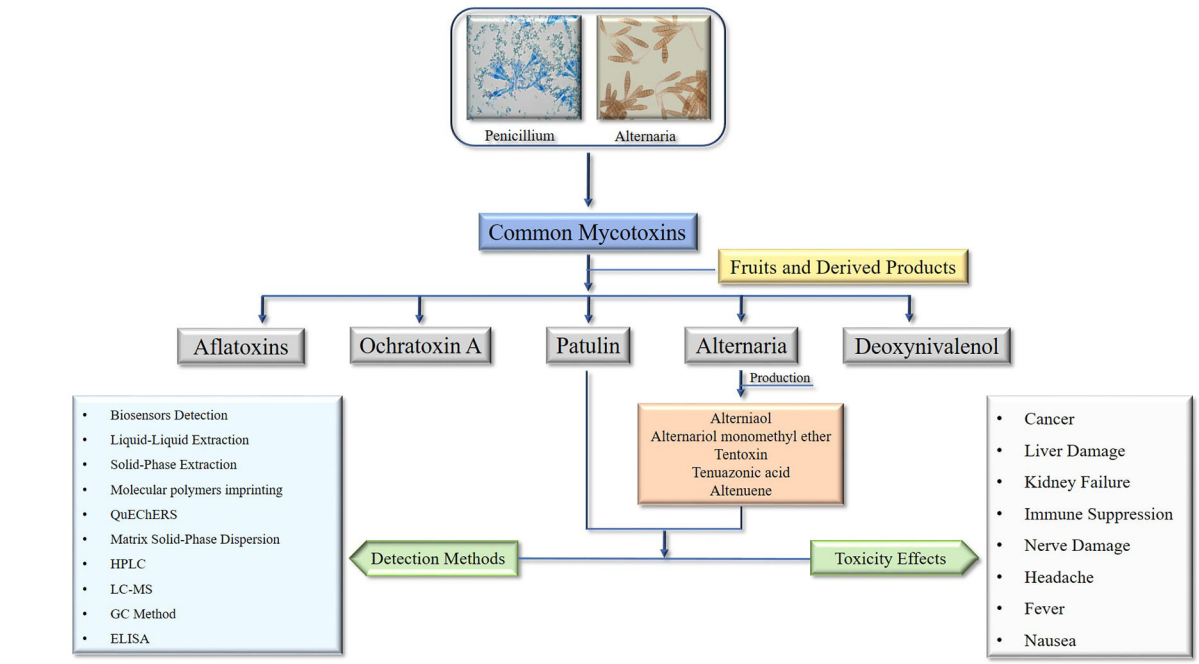
Syed Asim Shah Bacha[1], Yinping Li[1]*, Jiyun Nie[2]*, Guofeng Xu[1], Lingxi Han[2] and Saqib Farooq[1]

[1]Laboratory of Quality & Safety Risk Assessment for Fruit, Research Institute of Pomology, Chinese Academy of Agricultural Sciences, Xingcheng, Liaoning, China, [2]College of Horticulture, Qingdao Agricultural University/Laboratory of Quality & Safety Risk Assessment for Fruit (Qingdao), Ministry of Agriculture and Rural Affairs/National Technology Centre for Whole Process Quality Control of FSEN Horticultural Products (Qingdao)/Qingdao Key Lab of Modern Agriculture Quality and Safety Engineering, Qingdao, China

Mycotoxins are toxic secondary metabolites produced by certain fungi, which can contaminate various food commodities, including fruits and their derived products. Patulin and *Alternaria* toxins are among the most commonly encountered mycotoxins in fruit and their derived products. In this review, the sources, toxicity, and regulations related to these mycotoxins, as well as their detection and mitigation strategies are widely discussed. Patulin is a mycotoxin produced mainly by the fungal genera *Penicillium*, *Aspergillus*, and *Byssochlamys*. *Alternaria* toxins, produced by fungi in the *Alternaria* genus, are another common group of mycotoxins found in fruits and fruit products. The most prevalent *Alternaria* toxins are alternariol (AOH) and alternariol monomethyl ether (AME). These mycotoxins are of concern due to their potential negative effects on human health. Ingesting fruits contaminated with these mycotoxins can cause acute and chronic health problems. Detection of patulin and *Alternaria* toxins in fruit and their derived products can be challenging due to their low concentrations and the complexity of the food matrices. Common analytical methods, good agricultural practices, and contamination monitoring of these mycotoxins are important for safe consumption of fruits and derived products. And Future research will continue to explore new methods for detecting and managing these mycotoxins, with the ultimate goal of ensuring the safety and quality of fruits and derived product supply.

KEYWORDS

patulin, *Alternaria*, detection method, fruits, biosynthesis pathways, management strategies



GRAPHICAL ABSTRACT

1 Introduction

Fruits are essential nutritional sources for humans and a staple of the human diet. Mycotoxins contamination is a significant cause of production loss and a threat to consumer health and safety (Xue et al., 2010; Zhou et al., 2014; Rizzo et al., 2021). Due to their structural stability and resistance to heat, mycotoxins are difficult to remove during food processing and can ultimately persist in food and food products (Barkai-Golan and Paster, 2008; Oliveira et al., 2014). In certain countries, mycotoxin contamination can affect up to 50% of agricultural products, while in general, approximately 25% of agricultural products are affected by these toxins (Adeyeye and Oyewole, 2016; Graybill and Bailey, 2016). As a result, numerous countries and international organizations have established regulatory limits for mycotoxins (Li et al., 2020b; Notardonato et al., 2021). Even at low concentrations, mycotoxins exhibit a wide range of biological activities, including teratogenic, mutagenic, carcinogenic, and cytotoxic effects (Shi et al., 2019). As reports of food safety issues continue to increase, concerns over food safety problems resulting from mycotoxin contamination have grown among the public worldwide (Li et al., 2020b). Fungi produce low molecular weight mycotoxins, and although over 300 mycotoxins have been reported, only a limited number pose a threat to the health of humans and animals (Vejdovszky et al., 2017; Kermany et al., 2018). The most common mycotoxins in fruits and their derived products are patulin produced by *penicillium* species, *Alternaria* mycotoxins produced by *Alternaria* species (Fernández-Cruz et al., 2010; Li et al., 2020b).

Patulin is a significant mycotoxin found in various fruits, predominantly produced by *P. expansum* during storage. Its presence in baby food, including homogenized and fruit juices, is a major concern. Moreover, the International Agency for Research on Cancer categorizes patulin as a Group 3 substance, which means it has the potential to cause cancer, but there is currently insufficient evidence to confirm its carcinogenicity (Alshannaq and Yu, 2017; Notardonato et al., 2021). Although it has received relatively less attention compared to other mycotoxigenic fungi, *Alternaria* mycotoxin is a significant source of mycotoxins and can cause stem and leaf spot diseases, as well as spoil fruits and kernels during postharvest stages. As a result of its high prevalence in various food commodities and the presence of its toxins in fruits and their derived products, there has been a surge in scientific research on this fungal genus in recent years (Patriarca et al., 2019). Due to its ability to grow at low temperatures, the *Alternaria* genus is primarily responsible for the spoilage of numerous fruits, and their products during long-distance transport and refrigerated storage (Liang et al., 2016).

Alternaria and patulin, two types of more vivacious mycotoxins, can be found in fruits and fruit-derived products. These mycotoxins can be harmful to human health, and therefore their generation, accumulation, biosynthesis, and detection methods are of great interest to food safety researchers and practitioners (Mahakham et al., 2016). The generation and accumulation of *Alternaria* and

patulin mycotoxins in fruits and fruit-derived products are influenced by various factors, such as temperature, humidity, storage conditions, and the presence of other microorganisms. For instance, high humidity and warm temperatures are conducive to the growth of *Alternaria* and *Penicillium* fungi, which can lead to increased mycotoxin production (Gutarowska et al., 2015). The biosynthesis of *Alternaria* mycotoxins involves various enzymes, including polyketide synthases (PKSs), non-ribosomal peptide synthetases (NRPSs), and cytochrome P450 monooxygenases (CYPs) (Villena et al., 2020). The biosynthesis of patulin involves a complex pathway that includes several enzymatic reactions. Various methods are available for the detection of *Alternaria* and patulin mycotoxins in fruits and fruit-derived products, including: High-performance liquid chromatography (HPLC) that separates and quantifies mycotoxins based on their chemical properties, enzyme-linked immunosorbent assay (ELISA) that bind to specific mycotoxins and the presence of mycotoxins in a sample can be detected using colorimetric or fluorescence methods, mass spectrometry (MS) to identify and quantify mycotoxins based on their mass-to-charge ratio, and Biosensors that use biological components to detect mycotoxins in food products to detect specific mycotoxins or a range of mycotoxins (Freire and Sant'Ana, 2018). Subsequently, *Alternaria* and patulin mycotoxins are a concern in fruits and fruit-derived products due to their potential harmful effects on human health. Researchers and practitioners use various methods to detect and quantify these mycotoxins in food products. Understanding the factors that influence their generation and accumulation can help mitigate the risk of mycotoxin contamination.

In view of the great scientific importance of patulin and *Alternaria*, mycotoxins considered to be the most vibrant contaminants in fruits, this review is organized with the objectives to comprehensively evaluate the *Alternaria* and patulin mycotoxin generation in fruits and their derived products along with the accumulation, biosynthesis, and detection methods of these mycotoxins in fruit and derived products, to ensure food safety, improving quality control, and developing new products.

2 Mask and emerging mycotoxins

Mycotoxins are toxic compounds produced by fungi that grow on nuts, fruits, and their derived products. They can contaminate fruits and products and pose a health risk to humans and animals if ingested. Masked mycotoxins are a class of mycotoxins that are not readily detected using standard analytical methods. They are formed when a mycotoxin binds to other compounds in the food matrix, such as proteins or sugars, making it difficult to detect and quantify (Berthiller et al., 2013). However, these masked mycotoxins can be converted back into their active form in the body during digestion, potentially causing adverse health effects. Researchers are continuing to identify new mycotoxins and study their potential health effects (Grace et al., 2015). Emerging mycotoxins are newly discovered mycotoxins or those that have only recently been recognized as a potential health risk. They may not yet be regulated or monitored in food products, and their toxicity and prevalence are still being studied. Some examples of emerging mycotoxins include enniatins, beauvericin, and alternariol. Alternariol is a mycotoxin produced by several species of *Alternaria* fungi (Gruber-Dorninger et al., 2017). It has been found in a range of fruits and their products, and has been associated with genotoxic and immunotoxic effects (Gruber-Dorninger et al., 2017). One of the characteristics of *Alternaria* mycotoxins is that they can occur as "masked" mycotoxins. Masked mycotoxins are mycotoxin conjugates that are formed when mycotoxins bind to other molecules, such as sugars or amino acids, in the plant material. These conjugates are not usually detected by conventional mycotoxin analysis methods, as the mycotoxin is masked and not present in its free form (Freire and Sant'Ana, 2018). However, during digestion, the conjugate can be broken down, releasing the free mycotoxin. Masked *Alternaria* mycotoxins can pose a health risk, as they may not be detected by routine testing methods, and their potential toxicity is not well understood. The European Food Safety Authority (EFSA) has identified AOH and AME as potential masked mycotoxins of concern and has recommended further investigation into their occurrence and toxicity (Capriotti et al., 2010). In the case of patulin, it is a relatively stable compound and can be detected and quantified using standard analytical methods (Freire and Sant'Ana, 2018). While patulin is not widely considered an emerging mycotoxin, it is still a significant concern because of its potential health effects. Patulin has been associated with acute toxicity, including nausea, vomiting, and diarrhea, as well as long-term health effects such as immunotoxicity and genotoxicity (Barac, 2019b). However, it is important to note that patulin can be degraded during food processing or storage, which can result in the formation of other toxic compounds (Marin et al., 2013). Additionally, patulin can also interact with other compounds in food, which may affect its bioavailability and toxicity. Therefore, even though patulin is not considered a masked mycotoxin, it is still important to monitor and control its presence in food products (Arroyo-Manzanares et al., 2021).

Several methods are available for the detection of masked mycotoxins in food commodities. These include immunoassays, chromatographic methods, and mass spectrometry (Anfossi et al., 2016). Immunoassays are sensitive and relatively simple to perform, but they can produce false positive results. Chromatographic methods, such as high-performance liquid chromatography (HPLC), are commonly used for the detection of mycotoxins. These methods can separate the components of a sample and identify specific mycotoxins based on their retention time and spectral properties. Mass spectrometry is a powerful analytical technique used for the detection of mycotoxins. This method can provide high sensitivity, specificity, and selectivity, making it useful for the detection of masked mycotoxins (Singh and Mehta, 2020). Several studies have reported the presence of masked patulin and *Alternaria* mycotoxins in various food commodities. For instance, masked patulin was detected in apple juice, applesauce, and apple cider vinegar samples by using HPLC coupled with fluorescence detection (Li et al., 2020b). In another study, masked *Alternaria* mycotoxins were found in tomato sauce, chili powder, and paprika samples by using LC-MS/MS. The masking of mycotoxins in food

commodities can make their detection challenging. Patulin and *Alternaria* mycotoxins are two types of mycotoxins that can be masked, and they pose significant health risks to consumers. Several methods, including immunoassays, chromatographic methods, and mass spectrometry, are available for the detection of masked mycotoxins. Careful monitoring of food commodities is essential to prevent the exposure of consumers to these harmful mycotoxins (Iqbal, 2021).

3 Patulin

Patulin is a synthesized mycotoxin produced by several species of the genera *Aspergillus* and *Penicillium*, and it has been identified in many vegetables, cereals, moldy fruits, and other foods. Patulin has a low molecular weight, composed of alpha-beta unsaturated gamma lactone, which can contaminate many different foods, especially fruit and their products (Ngolong Ngea et al., 2020). It can produce citrinin, ochratoxin A, patulin, penitrem A, and rubratoxin B. Patulin has been identified in tomatoes, other fruit crops, and several consumer products, including dehydrated (dried) products (Zheng et al., 2018; Biango-Daniels et al., 2019; Saleh and Goktepe, 2019). Fruits contain a lot of water and sugar, which increases patulin activity (Iqbal et al., 2018; Zhong et al., 2018; Saleh and Goktepe, 2019; Solairaj et al., 2020). Patulin was a vital fruit concentrate (Zheng et al., 2018) in many juices, compote mixtures, commercial apple-based beverages, and baby foods (Zheng et al., 2018). Patulin has drawn global attention because it exacerbates health risks as it has mutagenic, carcinogenic, neurotoxic, genotoxic, immunotoxic, and gastrointestinal effects on human and animal health. In the 1960s, patulin was used for treating common colds and nose infections because of its antiviral, antiprotozoal and antibacterial properties. Later, it was classified as a true mycotoxin because of its toxic effects on human and animal health (Piqué Benages et al., 2013; Zaied et al., 2013).

Patulin also appeared to be severely hazardous in the post-harvest life of fruits, starting from single grain to the contamination of whole fruit and ending up spoiling the entire stored fruits (Hussain et al., 2020). Because of the blue mould decay and the subsequent production of patulin, the profitability of fruit producers has been jeopardized, as has human health (Cummings et al., 2018). It is one of the hazardous mycotoxins in fruits and induces a series of acute symptoms, gastrointestinal disturbances, vomiting, nausea, and chronic damage to the immune system, liver, and kidney (Barac, 2019a). Patulin is very stable in low values of pH near four, and ripened fruits are ideal for their production and biosynthesis. It is mainly found in apple fruit and processed products (Kumar et al., 2018).

3.1 Patulin producing fungi

Fungi produce the patulin in different fruits, including *Aspergillus clavatus*, *A. giganteus*, *A. terreus*, *P. coprobium*, *B. nivea*, *Paecilomyces variotii*, *P. clavigerum*, *P. concentri-cum*, *Byssochlamys fulva*, *P. dipodomyicola*, *P. expansum*, *P. roqueforti*, *P. sclerotigenum*, *P. vulpinum*, *Penicillium carneum* and *P. glandicola* (Bodinaku et al., 2019; Paramastuti et al., 2021). However, *P. expansum*, The source of blue mould in apples and apple-related products, is the most prevalent and significant patulin producer (Piqué et al., 2013; Zaied et al., 2013). Although the optimum temperature for *P. expansum* growth is 25°C, it can also survive at -3°C. Patulin production decreases as the temperature decreases up to freezing temperature (0-4°C), and moisture content must be in the range of 0.82-0.83 for spore formation (Sommer et al., 1974; Hasan, 2000). In addition, *P. expansum* can grow in low O_2 atmospheric concentrations and be found at as low as 2%. Furthermore, fruits' physical and chemical properties, such as strength, flesh firmness, skin thickness, sugar content, pH, and antimicrobial compound presence, also affect patulin formation.

3.2 Patulin contamination on fruits and derived products

Although patulin impacts a variety of foods, the most pervasive toxic effect was seen in apples, as investigated by numerous researchers. A problem for fruit and goods derived from the fruit is the poisonous metabolite patulin produced by *Penicillium expansum*, which can contaminate many different foods. As a result, solutions that are affordable and successful are required to get rid of patulin and ensure food safety. Consuming mouldy and seemingly clean but fungus-infected products increases the danger of consumers being exposed to patulin. This could be explained by the fruit's physicochemical properties, which encourage *P. expansum* growth and include factors like water activity and pH (Pleadin et al., 2019). Additionally, the genetic makeup of the crop, which affects its capacity for wound healing and susceptibility to infection, also impacts patulin production. In research by (Janisiewicz et al., 2008; Pleadin et al., 2019), Several global studies have been conducted to determine the levels of patulin contamination in apple juice and apple juice concentrates. While removing rotting or damaged fruit can decrease the amount of patulin in juices, it cannot be completely eliminated due to the diffusion of the mycotoxin into the fruit's nutritional components. Studies have shown that the highest concentrations of patulin are typically found within 1cm of the injured area (Marín et al., 2011). *P. expansum*, also known as blue mould, is the primary culprit responsible for the presence of patulin in decayed apples as well as apple-based products like juices, jams, and ciders. Additionally, patulin contamination can also occur in other fruits such as plums, peaches, strawberries, apricots, and kiwifruits. (Neri et al., 2010; Sadok et al., 2018). To regulate patulin contamination in apple products like juice, cider, and puree, the European Union has established a maximum limit of 50 μg/kg. A study conducted in 2016-2018 found that patulin levels in apple juice and apple-based baby food were below the EU limit in most samples, but some exceeded the limit in some countries, such as Poland and Italy (Gaugain et al., 2020). The FDA has established a guidance level of 50 μg/kg for patulin in apple juice and apple juice concentrate, similar to the maximum limit set by the European Union. A survey conducted in 2015-2016 found that the majority of the tested apple

juice samples (95%) were below the FDA guidance level, but some imported apple juice samples had higher levels (Biango-Daniels et al., 2019). Patulin contamination in fruits and their derived products has been thoroughly reported in China. A study conducted in 2015-2016 found that patulin levels in apple juice and apple puree samples from different regions of China ranged from below the detection limit to 189.0 μg/kg (Córdova et al., 2019). Similarly, previous studies also found that patulin levels in apple juice samples from Turkey ranged from below the detection limit to 258.2 μg/kg, with some samples exceeding the EU limit (Gottardi et al., 2016) (Table 1).

Several species of *Aspergillus* and *Penicillium* produce patulin. In apples, the apple-rotting fungus *P. expansum* is the primary producer of patulin (Şenyuva and Gilbert, 2008). Patulin was found mainly in apples and sometimes in fruits like pears, apricots, and peaches, and mainly in rotten fruit parts Turkish visibly moulded and dried figs (Drusch and Ragab, 2003; Cheraghali et al., 2005; Karaca and Nas, 2006; Şenyuva and Gilbert, 2008). Numerous surveys have been carried out globally to investigate the levels of patulin contamination present in apple and apple juice concentrates (Cheraghali et al., 2005; Murillo-Arbizu et al., 2009). While removing rotten or damaged fruit may reduce the amount of patulin in juices, it is not possible to completely eliminate this mycotoxin as it can spread to healthy parts of the fruit (Gudmundsson et al., 2009). Eating food products that are infected with fungus, even if they appear visually clean, can

TABLE 1 Worldwide natural occurrence of *Alternaria* and patulin mycotoxins in fruits and derived products.

Fruit & Products	Mycotoxin	Country	Positive samples/total%	Range μg/kg μg/L	References
Apple	*Alternaria*	China	29(27.88)	0.08–6	Li et al., 2020b
Apple juice	*Alternaria*		41	2.20–3.10	–
Apple James	*Alternaria*		17	4.42-10.10	–
Apple vinegar	*Alternaria*	China	34	14.5	Li et al., 2020b
Apricot juice	*Alternaria*	Germany	14/20	1.95–3.97	–
Grape juice	*Alternaria*		4	50	Zwickel et al., 2016
Citrus juice	*Alternaria*		1/1	2.04	–
Grape juice	*Alternaria*		7/8	1.58-6.44	–
Apples	*Alternaria*	Netherland	1/11	29	Sanzani et al., 2016
Fruits dried	*Alternaria*	Switzerland	9	<2–17.2	Mujahid et al., 2020
Tomato,	*Alternaria*	Belgium	23/27	<3.5-31	Gotthardt et al., 2019
Rotten mandarins	*Alternaria*	Italy	–	1000–5200	Logrieco et al., 2002
Fruit juices,	patulin	Iran	–	50	
Apple, juice	patulin	south Korea	3/24	2.8–8.9	Wie et al., 2010
Apple	patulin	Czech	5/6	3.8-28.4	–
Strawberry	patulin		0/3	>0.5	–
Pear	patulin		2/3	11.3-28.9	Veprikova et al., 2015
Apple juice	patulin	Qatar	20/20	5.8-82.2	Hammami et al., 2017
Peach	patulin	Italy	2/30	> 10	–
Pear	patulin		25/39	>10	Spadaro et al., 2008
Mixed juice	patulin	Tunisia	17/34	10/55.7	Vaclavikova et al., 2015
Apple with soya	patulin	Spain	37	49.9	Sargenti and Almeida, 2010
Pear marmalade	patulin	Argentina	1/6	44676	Sadok et al., 2019b
Salad of mixed fruits	patulin	Belgium	1/100	–	Van de Perre et al., 2014
Apricot	patulin	Thailand	4/10	6.3	–
Peach	patulin		3/8	5.6	–
Grape	patulin		7/18	3.5	Puangkham et al., 2017
Pineapple	patulin	Malaysia	1/17	33	–
Lychee juice	patulin		1/17	13	Lee et al., 2014

increase the risk of exposure to patulin for consumers. This is because mycotoxin can accumulate not only in the visible lesion but also in other parts of the spoiled fruit (García-Cela et al., 2012; Sanzani et al., 2016). It is important to monitor patulin contamination levels in fruits and related products, including juices, purees, ciders, jams, marmalades, vinegar, and dried fruits, and to establish accepted levels for these mycotoxins (Zhu, 2014; WANG et al., 2018). According to recorded literature, the concentration of patulin in apples that are infected with the toxin can range from 8.8 to 120.4 μg $kg^{-1.}$ Ritieni tested six samples of apple puree and found that three of them had patulin concentrations ranging from 15.9 to 74.2 μg kg^{-1}. However, only one of the samples met the European Union's standards for patulin levels, which stipulate that the mycotoxin should not exceed 25 μg kg^{-1} (Camaj et al., 2018). Four out of eight apple puree samples contained 22-221μg kg^{-1} of patulin, according to Funes and Resnik (Zhang et al., 2020b). In addition, the fungi that produce patulin contaminate other fruit products, Pears are also a source of this mycotoxin and have been found to have high levels of patulin, exceeding the recommended limit set by the European Union (Karlovsky et al., 2016a). Because the loss of patulin during industrial processing is relatively tiny, apple juices are mainly associated with patulin contamination (Zhang et al., 2020b). In China, patulin was detected in 19 out of 30 baby food products analyzed, with the maximum concentration of the mycotoxin reaching 67.3 μg/kg (Yuan et al., 2010). Other products from apples tested in Italy contained a significant amount of patulin. This emphasizes the importance of developing preventative actions and food surveillance programs to better protect children from toxin exposure. Patulin-producing fungi can contaminate various fruits other than apples, serving as another source of this mycotoxin. It is becoming more and more crucial to regularly check the levels of patulin present in colored fruits, such as hawthorns, red grapes, plums, sour cherries, as well as various types of berries like strawberries, raspberries, blueberries, and blackberries (Vaclavikova et al., 2015; Vickers, 2017; Iqbal et al., 2018; Wu et al., 2019).

3.3 Biosynthesis of patulin

The biosynthesis of patulin involves several enzymatic reactions that occur in the fungal cell. The first step is the condensation of two molecules of acetyl-CoA to form 6-methylsalicylic acid (6-MSA), which is catalyzed by the polyketide synthase (PKS) enzyme. Then, 6-MSA is converted into patulin by a series of reactions that involve oxidation, decarboxylation, and esterification reactions. Patulin is a secondary metabolite derived from polyacetate, and its metabolic pathway has been extensively studied utilizing cell-free extract and kinetic pulse-radiolabelling systems (Tian et al., 2018). The first limitation on patulin production has been identified as the inactivation of 6-MSA synthetase (Hitschler and Boles, 2019). Loss of 6-MSA synthetase is a selective process because *P. urticae* has a highly similar fatty acid synthetase (Moake et al., 2005). A study was conducted to investigate the stabilization of 6-MSA synthetase through the treatment of reaction mixtures containing nicotinamide adenine dinucleotide phosphate (NADPH) cofactor, Acetyl-CoA, and malonyl CoA with the reducing agent dithiothreitol and the proteinase inhibitor phenylmethylsulfonyl fluoride. The results demonstrated that the treatment effectively stabilized 6-MSA synthetase. This suggests that proteolysis and conformational integrity play a crucial role in 6-MSA synthetase regulation (Joshi et al., 2013). The activity of 6-MSA decarboxylase converts 6-MSA into m-cresol in the next stage of patulin biosynthesis (Kong et al., 2018). Further, m-cresol 2-hydroxylase converts m-cresol to m-hydroxy benzyl alcohol (Mahato et al., 2021). There is ongoing debate regarding the next step in the biosynthetic pathway of patulin, with two proposed mechanisms. However, both mechanisms agree that m-hydroxy benzyl alcohol eventually transforms into gentisaldehyde. Therefore, the conversion of m-hydroxy benzyl alcohol to gentisaldehyde is considered a crucial step in the patulin biosynthetic pathway (Yli-Manila and Gagkaeva, 2018). However, the intermediary between these two compounds is thought to be either gentisyl alcohol or benzyl alcohol (Lubbers et al., 2019). The exact mechanism of patulin biosynthesis in *P. expansum* is still under investigation. However, recent studies have identified several genes involved in patulin biosynthesis, including the *PKS* gene (*pksJ*), the oxidoreductase gene (*patG*), and the methyltransferase gene (*patK*). These genes are clustered together in the fungal genome and are regulated by a pathway-specific transcription factor (*patL*). Additionally, environmental factors such as temperature, pH, and nutrient availability can affect patulin production in fungal cells. For instance, studies have shown that low pH conditions and low temperatures can increase patulin production in *P. expansum*, while high temperatures and nutrient-rich conditions can decrease patulin production (Liu and Wu, 2010; Li et al., 2019) (Figure 1).

3.4 Health impact of patulin mycotoxin

The assessment of the health risks posed by patulin to human beings is based on many studies conducted over the past 50 years. Patulin causes various acute, chronic, and cellular levels in humans (Fung and Clark, 2004). Ingestion of patulin can lead to a range of adverse effects, including agitation, convulsions, dyspnea, pulmonary congestion, edema, ulceration, hyperemia, distension of the gastrointestinal tract, intestinal hemorrhage, degeneration of epithelial cells, inflammation of the intestines, vomiting, and damage to the gastrointestinal and kidney tissues (Celli et al., 2009; Pal et al., 2017; Aslam et al., 2021). Additionally, chronic health risks associated with patulin consumption include neurotoxic, immunotoxin, immunosuppressive, genotoxic, teratogenic, and carcinogenic effects (Abrunhosa et al., 2016; Boussabbeh et al., 2016; Ülger et al., 2020; Cimbalo et al., 2020; Ráduly et al., 2020).

Patulin has been associated with a range of health impacts, including neurotoxicity, immunotoxicity, and carcinogenicity. Patulin mycotoxin is recognized as a potential health risk worldwide, and regulatory agencies have established maximum limits for patulin in fruit products to ensure that consumers are

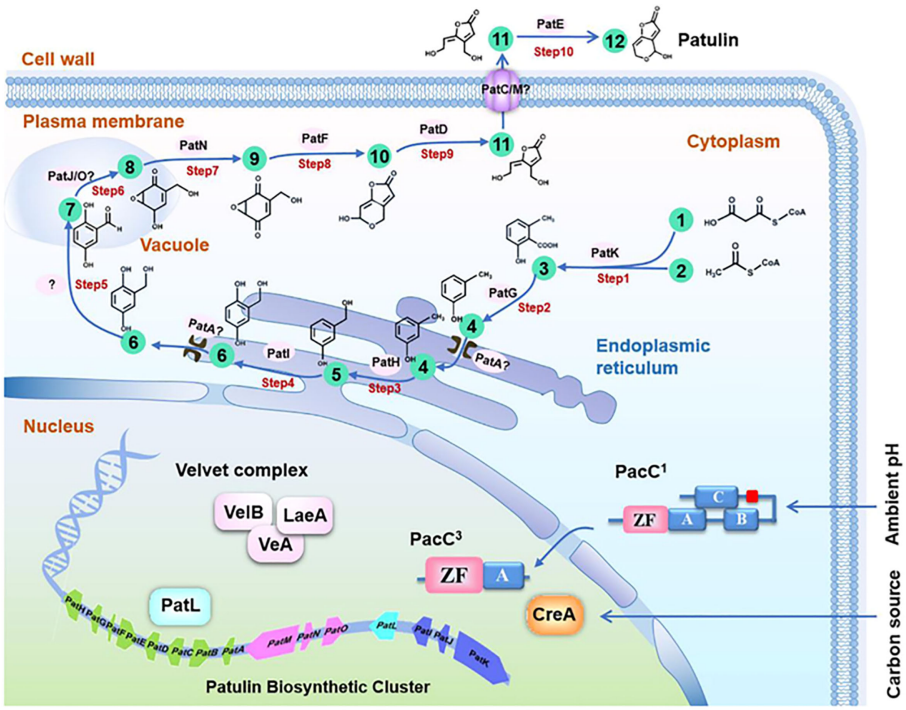


FIGURE 1
Molecular basis and regulation of the patulin biosynthetic pathway in *Penicillium expansum*. The patulin biosynthetic pathway consists of a 10-step reaction and 12 metabolites. 1, Acetyl CoA; 2, Malonyl CoA; 3: 6-Methylsalicylic acid; 4: m-Cresol; 5: m-Hydroxybenzyl alcohol; 6: Gentisyl alcohol; 7: Gentisaldehyde; 8: Isoepoxydon; 9: Phyllostine; 10: Neopatulin; 11: E-ascladiol; 12: Patulin. (Li et al., 2020a).

protected. Consumers should follow safe food handling practices, including checking for spoilage and discarding any fruits or juices that show signs of spoilage or mold growth. The incidence of patulin contamination is a global problem, mainly for the primary producers of apples and apple-based products, such as the USA, EU, and China (USDA, 2018). The Food and Drug Administration (FDA) of United States has set a maximum limit for patulin in apple juice and apple juice concentrate at 50 parts per billion (ppb). Exposure to high levels of patulin has been linked to liver damage and immunotoxicity in animal studies. However, the risk of adverse health effects from patulin in apple products is considered low for the general population, as most people consume these products in moderation (FDA et al., 2005). The European Commission has set a maximum limit for patulin in apple juice and apple juice concentrate at 50 ppb, the same as in the US. In addition, the EFSA has conducted risk assessments of patulin in other food products, such as pears, grapes, and dried fruits, and has concluded that exposure to patulin from these sources is also unlikely to pose a health risk for the general population (Capriotti et al., 2010). China has also set a maximum limit for patulin in apple products at 50 ppb. In recent years, there have been several reports of high levels of patulin in apple products in China, leading to concerns about food safety. The Chinese government has taken measures to improve the quality and safety of apple products, including strengthening regulations and increasing inspections of apple processing facilities (Capriotti et al., 2010). Health Canada has set a maximum limit for patulin in apple juice and apple juice concentrate at 50 ppb, consistent with the US and European limits. Health Canada has also conducted risk assessments of patulin in other food products, such as pears and cherries, and has concluded that exposure to patulin from these sources is unlikely to pose a health risk for the general population (Mendonca et al., 2020). The maximum allowable limit for patulin in apple juice and apple juice concentrate is set at 50 parts per billion (ppb) by the Australia New Zealand Food Standards Code, which aligns with the limits established in other nations. To ensure adherence to these regulations, the Australian government monitors the levels of patulin in apple products (Ghosh, 2014). Studies in India have shown that patulin can cause neurotoxicity, including oxidative stress and changes in neurotransmitter levels, and may also have genotoxic and carcinogenic effects. However, there is currently no specific regulatory limit for patulin in fruit products in India (Diao et al., 2019). A study conducted in Brazil found that patulin levels in apple juice samples were generally low, but higher levels were found in some samples. The study also found that patulin was not significantly associated with the occurrence of gastrointestinal symptoms in children (Dias et al., 2019). Similarly, a study conducted in Turkey found that patulin levels in apple juice and apple-based products were generally low, but higher levels were found in some samples (Table 2). The study also found that patulin was not significantly associated with the occurrence of gastrointestinal symptoms in adults (İçli, 2019).

Patulin has been shown to have cellular effects such as plasma membrane disruption (Li et al., 2019), protein synthesis inhibition of Na+-coupled amino acid (Schilling and Eder, 2004), disruption of transcription and translation (Rutkowski et al., 2015; Zhu et al.,

TABLE 2 Methods for detecting PAT using biosensors.

Biosensor Methods	Sensitivity/ LODs (µg/L)	Linearity (µg/L)	Pattern of Recognition	Mode/Type of Transmission	Reference
The use of ZnO nanorods on aptamer-based voltammetric patulin assay.	2.7×10^{-4}	5.0×10^{-4}-50	Self-assembled thio-modified aptamer complex on AuNPs	ZnO nanorods (ZnO-NRs) and chitosan composite modified gold electrode	Liu et al., 2018
Carbon dots, chitosan and gold nanoparticles have been added to a novel molecularly imprinted electrochemical sensor.	1.2×10^{-4}	5×10^{-4}–1.5×10^{-1}	Modified MIP cavity absorption	Electrochemical detection	Guo et al., 2017
Enzyme biosensor with conductometry	$<7.7 \times 10^{-2}$	$<1.5 \times 10^{-2}$-7.7×10^{-3}	Enzymes (Urease Inhibition)	Interdigitated gold electrodes in a differential pair	Soldatkin et al., 2017
Method of near-infrared fluorescence assay	< 6.0 × 10-2	0.0-3.7 × 10^{-1}	Anti-PAT antibodies conjugated to fluorophores	Fluorescence in the near infrared (NIR)	Pennacchio et al., 2015
Biochip based on surface plasmon resonance	1.5×10^{-2}	0.0-12.3	Antibodies compete with anti-patulin	Resonance of surface plasmons (SPR)	Vickers, 2017
A colorimetric DNA apt sensor.	4.8×10^{-2}	5.0×10^{-2}–2.5	DNA aptamer	Colorimetric detection	Jin et al., 2019
Immune system-based sensor (biosensor) on nanoporous silicon sensor (biosensor)	–	–	Immune system based on nanoporous silicon	Single-crystal silicon square wafers doped with boron	Starodub and Slishek, 2013
Aptasensor Impedimetric	2.8×10^{-3}	25×10^{-3}–1	A carbon-based electrode -immobilized aptamer interaction	Electrochemical detection	Khan et al., 2019
Molecularly imprinted electrochemical sensing platform (thionine)	1.0×10^{-3}	2.0×10^{-3}–2	Thianine tailing surface	Electrochemical detection	Khan et al., 2019
Quartz microbalance sensor based on molecular sol-gel polymer (MIP)	3.1	7.5-60	Sol-gel molecular polymer (MIP) Changing QCM frequency	–	Chen et al., 2016
Surface-impressed gold Surface-enhanced nanoparticles Scatter sensor (MIP-SERS) Sensor) Sensor	8.3×10^{-4}	1.1×10^{-3}–7.7	Selectivity of molecular polymer imprinted	Electrochemical detection	Wu et al., 2019
Molecularly imprinted polymer surface Capped ZnS quantum dots Mn-doped as a nanosensor phosphorescent	49.3	1.0×10^{-3}–66.3	6-hydroxynicotinic acid affinity (6-HNA)	Detection of phosphorescence quenching	Zhang et al., 2017b

2019), inhibition of DNA synthesis (Varzatskii et al., 2018) as well as the inhibition of T-helper type 1 cells that produce interferon (Chai et al., 2010). Patulin damages cells by forming adducts with thiol-containing cellular components like glutathione and cysteine-containing proteins (Pal et al., 2017). Patulin is toxic to many enzymes with a sulfhydryl group in their active site. A recent study revealed that patulin inhibits ATPase, which is Na+ K+ dependent RNA polymerases (Lee et al., 2019) and the synthetase of aminoacyl tRNA (Cervettini et al., 2020). Furthermore, exposure to patulin results in the loss of free glutathione in living cells (Pal et al., 2017). Recent research revealed that the exogenous cysteine and glutathione treatment prevents patulin toxicity in the intestinal epithelium (Saleh and Goktepe, 2019). Patulin has also been shown to promote intramolecular and intermolecular protein cross-linking. This reaction favours cysteine's thiol group, but it also happens with lysine and histidine's side chains and -amino groups (Qiu et al., 2020). Other studies have also observed patulin's reactivity with NH_2 groups (Plunkett et al., 2019). Patulin has also been shown to inhibit protein prenylation, a necessary posttranslational protein modification involved in activating many proteins, including many oncogenes, such as Ras, that must be prenylated to function appropriately (Zheng et al., 2017).

4 *Alternaria* mycotoxins

The *Alternaria* genus was first described in 1816 (Woudenberg et al., 2013). *Alternaria* species belong to the phylum Ascomycota, commonly called sac fungi. It is saprophytic and parasitic and can decompose organic matter largely. It may also become an opportunistic pathogen sometimes causing various diseases in cereal crops, ornamentals, vegetables, and fruits (Thomma, 2003).

Alternaria spp. produce toxins as secondary metabolites that develop cancer and are mutagenic, causing health disorders in animals and humans (Pastor and Guarro, 2008). Moreover, its spores are airborne allergens that are problematic in certain situations (Kilic et al., 2020). Besides, *Alternaria* pathogenic species cause blight, leaf rot, and leaf spot diseases in plants associated with both host-specific and non-host-specific toxins (Yamamoto et al., 2014; Meena and Samal, 2019), causing black spots in various vegetables and fruits during the post-harvest period of storage and marketing (Thomma, 2003). *Alternaria* mycotoxins are considered emerging mycotoxins because they have been increasingly recognized as a potential health hazard in recent years. *Alternaria* fungi are known to produce a wide range of mycotoxins that can contaminate a variety of fruits and their products. Exposure to these mycotoxins has been associated with various adverse health effects, including cancer, allergies, and other toxicities. The classes of *Alternaria* mycotoxins include, alternariol (AOH), alternariol monomethyl ether (AME), tentoxin, altenuene, alternapyrone, and aurasperone A. Several studies conducted on the occurrence of *Alternaria* mycotoxins in fruits in different countries. Studies conducted in Italy found that *Alternaria* mycotoxins were present in a variety of fruits, including apples, pears, and grapes. The most common mycotoxins detected were AOH and AME (Zhou and Hu, 2018; Ye et al., 2019). A study in China found that *Alternaria* mycotoxins were present in a variety of fruits, including apples, pears, and peaches. The most common mycotoxin detected was AOH (Ji et al., 2022). A study in Iran found that *Alternaria* mycotoxins were present in pomegranates, with the most common mycotoxin detected being tentoxin (Vidal et al., 2019). A study in Spain found that *Alternaria* mycotoxins were present in strawberries, with the most common mycotoxin detected being AME. A study in Brazil found that *Alternaria* mycotoxins were present in mangoes, with the most common mycotoxin detected being AOH (Qiao et al., 2018; Cinar and Onbaşı, 2019). It is also important to note that the occurrence of *Alternaria* mycotoxins in fruits can vary depending on a number of factors, including the type of fruit, the location where it was grown, and the environmental conditions during cultivation and storage (Table 1).

4.1 *Alternaria* mycotoxins-producing fungi

The naturally occurring secondary metabolites known as "*Alternaria* mycotoxins" are produced by toxigenic micro-fungi that grow on crops (Nesic et al., 2014; Tralamazza et al., 2018). Different species of *Alternaria* can produce mycotoxins, including *A. scripinfestans*, *A. botrytis*, *A. oudemansii*, *A. leptenallea*, and *A. alternata* (Buczacki, 2003). The most common and important species is *A. alternata*, which produces mycotoxins and grows on cereal crops, vegetables, fruits, olives and sunflower seeds (Scott, 2001; Scott, 2004). Some other species were also called etiologic agents, including *A. tenuissima*, *A. longipes*, *A. infectoria*, *A. dianthicola*, and *A. chlamydospora* (Bartolome et al., 1999; Ferrer et al., 2003; Robertshaw and Higgins, 2005; Nulens et al., 2006). The morphology of these species is different yet challenging to identify and is a pretty daunting task to do (Sutton et al., 2009). To minimize the risk of exposure to *Alternaria* mycotoxins, it is important to store food properly, discard any moldy or damaged food, and avoid consuming foods that are known to be contaminated with these toxins. Additionally, agricultural practices that promote healthy plant growth and reduce fungal contamination can help to prevent the growth of *Alternaria* fungi and the production of mycotoxins in crops.

4.2 *Alternaria* mycotoxins contamination on fruits and derived products

Alternaria can produce mycotoxins, which are toxic compounds that can contaminate food and feed products (Müller et al., 2018). The mycotoxins produced by *Alternaria* toxin can cause adverse health effects in humans and animals if ingested in sufficient quantities. One of the most commonly produced *Alternaria* mycotoxins is AOH, which has been detected in various fruits and their derived products such as apple juice, grape juice, tomato sauce, and dried fruits (Solfrizzo, 2017). AOH has been shown to be genotoxic, carcinogenic, and immunosuppressive in animal studies, and its presence in food products has raised concerns about its potential health effects on humans. Another *Alternaria* mycotoxin that has been found in fruits and their derived products is alternariol monomethyl ether. AME has been detected in apple juice, grape juice, tomato products, and dried fruits. Like AOH, AME has been shown to be genotoxic and carcinogenic in animal studies. Other *Alternaria* mycotoxins that have been detected in fruits and their derived products include TeA, ALT, and ATX-I. TeA has been found in apple juice, grape juice, and tomato products, while ALT and ATX-I have been detected in apple products (Houbraken et al., 2010). Much research revealed that *Alternaria* pathogenicity is higher in fruits and leaves than in other plant parts (Harteveld et al., 2014). Secondary metabolites of *Alternaria* spp. may cause other plant diseases such as black tomato mould, the grey/black mould of citrus, olive black rot, apples, and carrot black rot (Logrieco et al., 2009). The presence of *Alternaria* mycotoxins in fruits and their derived products is a concern for food safety and human health. To mitigate the risk of mycotoxin contamination, it is important to implement good agricultural and manufacturing practices, including proper storage and handling of fruits and their derived products, as well as monitoring and testing for mycotoxin contamination (Li et al., 2020b).

Alternaria fungi are parasitic on plants and may cause fruit and vegetable spoilage during transportation and storage. *A. Alternata* is capable of producing a range of mycotoxins, such as alternariol dibenzo-α-pyrones, tenuazonic acid (a tetramic acid), ATX-I and II, monomethyl ether, and altenuene. It is important to note the variety of mycotoxins produced by *A. Alternata* (Yang et al., 2014). *Alternaria* toxins AME and AOH can be produced within a range of 5-30°C and a water activity (aw) range of 0.98-0.90. However, at the lower end of this a_w range (i.e., 0.90), very few mycotoxins are

produced. The minimum aw required for *A. alternata* conidia to germinate is 0.85. In contrast, wheat growth requires an a_w of at least 0.88 in extract agar at 25°C. As a result, the limiting aw for detectable mycotoxin production is slightly higher than for growth, with optimum production above 0.95 a_w (Magan et al., 1984). *Alternaria* mycotoxins can be found in various fruits, vegetables, and grains (Scott, 2001). AOH and AME are among *Alternaria* main mycotoxins, naturally reported as occurring in different fruits that are infected, including mandarins, oranges, melons, apples, lemons, and various berries (Scott, 2001; Drusch and Ragab, 2003; Scott et al., 2006). Tenuazonic acid was also present in these citric at high levels (Magnani et al., 2007). Monomethyl ether alternariol and alternariol were detected in tangerines in Brazil with and without symptoms of spot disease *Alternaria*; levels of these flavedo mycotoxins varied between 0.90 and 17.40 μg/kg Table 1. In albedo (mesocarp) tissues, neither AOH nor AME has been detected, suggesting that the flavedo is working as a barrier to these substances. The natural occurrence of *Alternaria* toxins in processed foods interests from a human health point of view. AOH and AME were detected in most of the fruit's juices with shallow (<1.5 μg/L) levels, except for apple, grape juice and red wine (Drusch and Ragab, 2003; Scott et al., 2006). The natural occurrence of AOH and AME in apple juice was reported at levels ranging from 0.04 to 2.40 μg/L and 0.03 to 0.43 μg/L Table 1t1, respectively; other fruit juices, such as grape juice, had levels of 1.6 and 0.23 μg/L for AOH and AME, respectively, 5.5 and 1.4 μg/L for prune nectar, and 5.6 and 0.7 μg/L for cranberry nectar low levels of raspberry juice have also been detected (Lau et al., 2003). Both mycotoxins have been found in apple juice concentrates from Spain 50% of the samples were analyzed as natural contaminants. Levels of the AOH were in the range of 1.35–5.42 μg/L. AME was present in most cases only at trace levels, and the highest number detected in one sample was 1.71 μg/L (Table 1). AOH occurs very often at low levels in red wine (Scott et al., 2006) AOH was found in 13/17 Canadian red wines at a level of 0.03–5.02 μg/L and in 7/7 imported red wines at a level of 0.27–19.4 μg/L, Table 1 accompanied by lower AME concentrations. White wines contained a small amount of AOH/AME (≤1.5 ng/mL). As far as we know, there are no studies on the co-occurrence of *Alternaria* toxins in fruit with other mycotoxins.

4.3 Biosynthesis of *Alternaria* mycotoxin

The biosynthesis of *Alternaria* toxin metabolites is a complex process involving many enzymes and pathways. One well-studied example of *Alternaria* toxin metabolite biosynthesis is the production of the mycotoxin AOH. AOH is a hydroxylated form of the precursor AME and is synthesized through a series of enzymatic reactions. The biosynthesis of AOH in *Alternaria* toxin involves a *PKS* gene cluster, which includes genes for a *PKS*, a ketoreductase (KR), an enoyl reductase (ER), and a cytochrome P450 monooxygenase (CYP). The *PKS* gene cluster produces a polyketide intermediate that is subsequently modified by the KR and ER enzymes to form the AOH precursor, AME. Finally, the CYP enzyme catalyzes the hydroxylation of AME to produce AOH. The biosynthesis of AOH has been extensively studied in *A. alternata*, and the *PKS* gene cluster responsible for AOH biosynthesis has been identified and characterized (Darma et al., 2019). Consequently, *Alternaria* toxin metabolite biosynthesis is the production of tenuazonic acid (TeA), a mycotoxin produced by many species of *Alternaria*. TeA is synthesized from a precursor molecule, 1,8-dihydroxynaphthalene (DHN), through a series of enzymatic reactions. The biosynthesis of TeA in *Alternaria* toxin involves a nonribosomal peptide synthetase (NRPS) gene cluster, which includes genes for an *NRPS*, a *PKS*, and a thioesterase (TE) (Yun et al., 2015). The NRPS enzyme catalyzes the formation of a peptide bond between two amino acids, while the PKS enzyme produces a polyketide intermediate. The TE enzyme cleaves the peptide-polyketide intermediate, leading to the formation of DHN, which is then modified to form TeA. The biosynthesis of TeA has been studied in several species of *Alternaria*, including A. *alternata*, and the *NRPS* gene cluster responsible for TeA biosynthesis has been identified and characterized (Yun et al., 2015) (Figure 2).

4.4 Health impact of *Alternaria* mycotoxin

The toxicity of *Alternaria* toxins in animals and humans has received insufficient attention compared to other commonly reported mycotoxins. While AOH and AME are not particularly acutely toxic to animals but can still cause organ hemorrhages, some animal species, like dogs, are highly toxic to TeA. For instance, they generate internal haemorrhage in the chicken while they reduce feed efficiency in mice (Agriopoulou et al., 2020). Three mammalian regular cell lines, 3T3 mouse fibroblasts, Chinese hamster lung cells, and human hepatocytes are the most sensitive to TeA cytotoxic effects on cultured cells, which include lowering total protein concentrations and suppressing proliferation (Song et al., 2020). AME and AOH are genotoxic, though previous research has shown that AOH is more genotoxic than AME in cultured human colon carcinoma cells while causing fetal oesophagal squamous (Aichinger et al., 2017). Even though individual mycotoxin concentrations are usually within permissible limits, high co-contamination rates could harm human and animal health. It is crucial to thoroughly evaluate the toxicological traits of individual mycotoxins and co-occurring mycotoxins. *Alternaria* mycotoxins produced can be harmful to human health and to ensure the safety of fruits and their derived products, many countries have established regulations on the maximum permitted limits of *Alternaria* content in these products (Table 2).

5 Detection technology of patulin and *Alternaria* mycotoxins in fruits and derived products

5.1 Biosensors detection

Patulin in different food commodities has historically been determined using several chromatographic techniques, such as gas chromatography, high-performance liquid chromatography, thin-

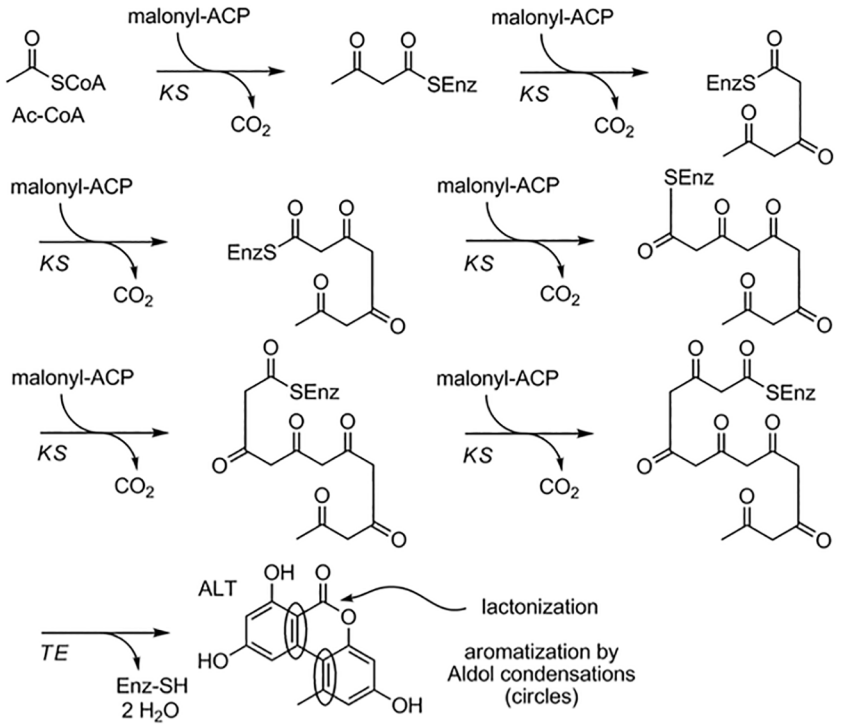


FIGURE 2
Proposed biosynthetic pathway for alternariol and alternariol-9-methyl ether.(Saha et al., 2012).

layer chromatography, and others, have been used to analyze patulin levels in different food commodities (Kaur and Singh, 2020). These are sensitive and targeted methods. However, they require costly equipment and highly skilled operators (Kaur and Singh, 2020). Biosensor technologies, as opposed to chemical methods, have recently been developed to provide some "cleaner" patulin detection techniques for apple juice. Selectivity, which allows for immediate detection of the analyte with little or no pretreatment, is an undeniable advantage of biosensors over traditional food analysis methods. They do not necessitate highly trained personnel and are simple to operate (Ngolong Ngea et al., 2020). Biosensor technologies use a specific bio-recognition component and a transducer to process the signal. The affinity of the bio-recognition component to the patulin molecule will determine its efficacy. The sensitivity of biosensors will depend on their capacity to detect even the weakest modification signal, usually an electrochemical signal, following patulin bio-recognition. We have included some recent examples of biosensors below. The competitive immunoassay is an intriguing method for investigating patulin in food. An innovative strategy has been developed by combining immunological recognition of patulin with a surface plasmon resonance optical procedure to create polyclonal antibodies (SPR) (Tittlemier et al., 2021). A laser beam initiated interactions between the test and targeted molecular particles on the biochip's gold surface. This induction causes a shift in resonance conditions and, as a result, a subtle but noticeable change in reflectivity. This method was described as a cost-effective and efficient immunoassay for determining patulin. According to Vickers' study on apple juice, the LODs in this test was found to be 1.54 μg/L. It should be noted that patulin was not detected prior to the sampling process (Vickers, 2017). A new fluorescence polarization method using near-infrared (NIR) fluorescence sensors was also been developed that showed a great potential in fluorescence detection and measurement (Víctor-Ortega et al., 2013; Pennacchio et al., 2015). The increase of emissions of fluorescence polarization of a fluorescence-labelled patulin derivative, which binds to particular antibodies, is characterized. The LODs for the patulin ranges from 6 to 102 μg/L due to competition between the patulin and the fluorescence-labelled patulin derivative (Melinte et al., 2022). The proposed technique was based on the unique properties of crystal or quartz materials. Oriented antibodies that were tethered were immobilised using photonics on the gold-plated surface of a quartz-equipped microbalance. This biosensor identified patulin at a concentration of 21.56 μg/L. An extra antibody was added in a "sandwich procedure" to enable detection of nano-sized analytes measured by a microbalance. Furthermore, the goal is to develop a simple luminescent sensor capable of detecting patulin. Further, Zhang et al. (2017b) have created a nano-sensor based on manganese-doped ZnS quantum dots that use phosphorescence to discriminate patulin selectively. With a LODs of 49.31 μg/L, this nano-sensor can detect patulin from a concentration range of 66.22 to 1.001 μg/L. It can also differentiate patulin from other mycotoxins. Much of the recent research has focused on the problem of restoring biosensor activity after it has been used. As a result, Soldatkin et al. (2017) tracked patulin inhibitory action, and researchers created a conduct metric urease-based biosensor. This biosensor is suited for assessing patulin concentrations beyond 50 μg/L in apple juices because it has

a relatively high patulin sensitivity, strong selectivity, and great signal repeatability. The presence of heavy metals, on the other hand, causes some problems. Patulin and other heavy metals can create strong covalent interactions with enzyme sulfhydryl groups. An oligonucleotide aptamer is a monoantennary DNA (or RNA) sequence. Aptamers are typically selected using a well-known method. The acronym SELEX (systematic evolution of ligands) stands for "systematic evolution of ligands." There are ssDNA aptamers present (by exponential enrichment). It generally has a high affinity for patulin and interesting properties like easy synthesis and labelling, no immunogenicity, low production costs, high stability, affinity, and outstanding specificity in target binding. This chosen aptamer was later used as a selective component in a patulin detection method based on a polymerase chain reaction. Enzymatic substrate system with chromogenic properties. The result was that the colourimetric aptasensor provided a linear response, which was very impressive. The detection range is 5 102 to 2.5 μg/L, and the detection range is 5 102 to 2.5 μg/L. The limit was 4.8 102 μg/L (Wu et al., 2015). Lanthanide-doped rare earth-doped up-conversion nanoparticles (UCNPs) have gotten much attention as a technique to increase biosensor signal transmission (Loo et al., 2019). NIR-to-visible up-conversion nanoparticles (UCNPs) offer several advantages over traditional down-conversion luminescent devices. These include low auto-fluorescence background for improved signal-to-noise ratio, high photostability, low toxicity, high Stokes offsets, tunable fluorescence wavelength, and deep tissue infiltration. Biosensors linked to a transducing system that uses biological tools like enzymes, aptamers, and antibodies for recognition are employed to detect patulin in food. Aptamers are gaining popularity due to their remarkable ability to recognize patulin at low concentrations and modify their absorption properties, enabling detection at extremely low levels. They can be useful for on-line patulin control in the food industry. The main challenges of biosensors are the limited stability of the bio-recognition component (which affects the biosensors' long-term storage stability), poor selectivity, especially in enzyme inhibition-based biosensors, and the high cost of antibodies when compared to synthetic recognition elements (Kaur et al., 2015; Burcu Aydın et al., 2020) (Table 3).

5.2 Molecular detection methods

Controlling patulin requires early detection of it-producing fungi (Mahunu et al., 2016). *Aspergillus clavatus*, *Aspergillus longivesica*, *Aspergillus* species, and *Aspergillus giganteus* are among the fungi that produce patulin. In the *Penicillium* genus, there are 13 species that produce patulin, including *P. vulpinum, P. sclerotigenum, P. paneum, P. marinum, P. griseofulvum, P. gladioli, P. glandicola, P. dipodomyicola, P. expansum, P. coprobium, P. concentricum, P. clavigerum, and P. carneum* (Mahunu, 2017). Polyphasic analysis of all *Byssochlamys* and related *Paecilomyces* species revealed that only *Byssochlamys Nivea* and a few strains of *Paecilomyces saturatus* could produce patulin (Fontaine et al., 2016). Given a large number of producers, biotechnology methods that identify the presence of patulin-producing fungi can assist in identifying the crucial areas that need to be controlled. PCR-based methods for detecting patulin -producing fungi strains in food samples can be used as a standard approach in agri-food HACCP procedures. The *IDH1* gene encodes the isoepoxydon dehydrogenase enzyme, which is required for patulin biosynthesis. In general, detection PCR protocols use high sensitivity and specificity to amplify the IDH1 sequence found in the genomes of some fungi species, such as *P. expansum* (Niessen, 2007; Delgado et al., 2021). However, some ingredients in complex foods inhibit PCR. These substances inhibit polymerase activity, which is necessary for DNA amplification, and cellular lysis, which is necessary for DNA extraction, capture, or degradation. Fortunately, a suitable nucleic acid extraction protocol could overcome this constraint (Aparicio et al., 2019). Regarding sensitivity, Delgado et al. (2021) developed RTi-PCR tests based on the *IDH1*1 gene for quantifying patulin-producing molds. When used in food, the newly developed RTi-PCR SYBR Green and TaqMan probes demonstrated significant sensitivity. Both RTi-PCR methods detected ten conidia per gram of food matrices, with an excellent linear relationship between the number of *IDH1* gene units and Ct values (Ngolong Ngea et al., 2020). As a result, real-time quantitative PCR (qPCR) has been shown to detect and quantify toxic molds in food before mycotoxin production" Similarly, Crespo-Sempere et al. (2013) developed a sensitive and efficient TaqMan qPCR multiplex method for quantifying fungi that produce mycotoxins and patulin. In order to generate PCR products specific to *B. nivea*, a PCR using conventional primer sets (B. nivea1F/1R) was completed (Kizis et al., 2021). Similarly, other amplification reactions using the standard primer sets B. lag 1F/1R, B. fulva 1F/1R, and B. zol3 F/R produced PCR products specific to *B. zollerniae*, *B. fulva*, *B. laguncularіae*, and *B. laguncularіae*, respectively. Early detection is essential for figuring out the crucial steps to get rid of moulds that produce patulin and, as a result, limit patulin in food. Early identification of patulin producers in food using PCR techniques will help stop patulin contamination after food production. Primers created from patulin biosynthesis genes are now readily available, and PCR and real-time PCR-based molecular techniques are evolving quickly. The existing primers, which were developed based on the search for patulin biosynthetic pathways in all potential patulin producers, not solely *P. expansum*, are not specific to all fungi that produce patulin.

5.3 Liquid-liquid extraction method

The extraction process is the initial stage in food analysis. It affects the target compound's purity and concentration in the extracted sample. LLE is one of the most widely used methods for extracting compounds. It involves using two solvents that are either immiscible or partially miscible with each other to separate one or more species. This technique has been employed in the analysis of aqueous samples in various studies, including mycotoxin detection in different foods. For HPLC-UV analysis of apple juice and apple puree, the Association of Official Analytical Chemists (AOAC) suggests utilizing LLE with ethyl acetate, followed by cleanup using sodium carbonat. This method (MacDonald et al., 2000)

TABLE 3 Comparison of Liquid liquid extraction conditions used for fruit commodity preparation for patulin analysis.

Application	Sample size	Extraction solvent volume	Organic phase cleanup	Additional steps	Recovery spiking level	Reference
Fruit juice	50g[a]or 50mL	50 mL of EtAC (15 min)2 × 20 mL of EtAC	2 mL of 1.5% Na2CO3 + 5 mL of EtAC (5 min)	Filtration, evaporation, and solvent reconstitution	85.5–93.7% (50.0–200.0 µg mL^{-1})	Iqbal et al., 2018
Apple juice	5mL	20 mL of EtAC (15 min)	–	Reconstitution of the solvent, Evaporation	77.0–113.0% (10.0–1000.0 µg kg^{-1})	–
Fruit juices,	–	–	–	–	–	Hammami et al., 2017
Apple juice,	5 mL or 5 g[a]	0 mL of EtAC (10 min);2 × 20 mL of EtAC	2 mL of 1.5% Na_2CO+ 5 mL of EtAC (3 min)	Solvent, evaporation filtration, reconstitution	86.5%(100.0–300.0 µg mL-1)	Zouaoui et al., 2015
Concentrate,	–	–	–	–	–	–
Appple jam	–	–	–	–	–	–
Apple juice	10mL	3 × 20 mL of EtAC(3 × 1 min)	4 mL of 1.5% Na_2CO_3+ 10 mL of EtACb	Filtering, evaporation, reconstitution of a solvent	86.0% (50 ug L^{-1})	
Juices of fruit,	10mL	3 × 20 mL of EtAC(3 × 10 min)	2 mL of 2% Na2CO3	SPE clean-up, evaporation solvent reconstitution	96.1–115.7% (20.0–50.0 µg L^{-1}	Zaied et al., 2013
Fruit juices	5mL	2 × 10 mL of EtAC(2 × 1 min)	2 mL of 1.5% Na2CO3+ 5 mL of EtACb	Filtration	70.0–82.0%(50.0–150.0 µg L^{-1}	Cho et al., 2010
Apple cloudy juice,	10g[a]	10 mL of EtAc: n-hexane(60: 40, v/v) (5 min)	15.0 g Na2SO4, 2 g $NaHCO_3$	Clean-up of SPE, evaporation, solvent reconstitution, and filtration	53.0–74.0%(5.0–48.0 µg kg^{-1})	–
Apple puree,	–	–	–	–	–	Režek Jambrak et al., 2018
Apple clear, Concentrated juice	5g[a]	3 × 25 mL of EtAC(3 × 1 min)	10 mL of 1.5% Na2CO3(10 s) + 10 mL of EtAC (1 min) b	solvent reconstitution, evaporation	75.2–89.2%(10.0–500.0 µg kg^{-1})	Malachová et al., 2014

was recently used to extract mycotoxin from apples and other fruit juices, with or without some modifications (Shephard and Leggott, 2000; Tamura et al., 2011; Malachová et al., 2014; Alshannaq and Yu, 2017; Iqbal et al., 2018). Unfortunately, because patulin is more stable in acidic media, adding sodium carbonate to the sample raises the pH and causes patulin degradation. The issue can be avoided by using different salts, like sodium sulfate and sodium hydrogen carbonate, as alternative SPE cleanup procedures (Liao et al., 2019). LLE has the disadvantage of requiring many organic solvents and taking a long time to prepare samples for analysis. To extract patulin from apple-based products like apple juice concentrate, mixed apple juice, and baby foods, the method involves using 25 mL of ethyl acetate to process 10 g of the sampled. After shaking vigorously for 3 minutes and centrifuging for 5 minutes, 20 mL of ethyl acetate is used to extract the aqueous phase twice. Sodium carbonate is added three times (in 2 mL portions) followed by shaking, and then one 5 mL portion of ethyl acetate is added.

The samples underwent chromatographic analysis after being processed through the following steps: pH adjustment, evaporation to dryness, reconstitution, and filtration (Sieper et al., 2019). Although this approach has shown to have substantial analyte recuperation rates in apple juice, achieving 92.0% and 98.0% recoveries for 10 µg kg^{-1} and 100 µg kg^{-1} of patulin spiking, respectively, it is widely recognized that it is a time-intensive method that demands a considerable amount of data. Moreover, the method generates a substantial amount of ethyl acetate waste (70 mL per sample), which can pose a danger when produced in large amounts (Yap, 2015). To extract patulin from a 10 g sample, it was diluted in 10 mL of water and subjected to three rounds of extraction using 50 mL, 25 mL, and 15 mL of ethyl acetate, respectively. After shaking for 10 minutes and centrifuging for 5 minutes, 9 mL of sodium carbonate solution was added, and the organic phase was agitated. The lower layer (sodium carbonate solution) was then extracted with 10 mL of ethyl acetate. The combined organic layer underwent dehydration, evaporation, reconstitution in a solvent, solid-phase extraction (SPE) purification, and filtration before chromatographic analysis. The method achieved satisfactory patulin recoveries (with a mean of 95%) when tested on baby food matrices spiked with mycotoxin at concentrations of 5-20 µg L^{-1}. However, the drawbacks of this technique include the use of a large amount of ethyl acetate per sample (100 mL) and the extended time required for sample preparation before chromatographic analysis. Recent developments in LLE have introduced an intriguing alternative to traditional LLE methods.

Ultrasonic techniques have emerged as a promising alternative for pre-treating juices and other fruit matrices for patulin determination using LLE (Gavahian et al., 2020). This method involves utilizing a blend of two solvents that do not mix with each other, namely the water present in the fruit matrix and an organic solvent. The approach enables the extraction of several samples simultaneously, while minimizing the quantity of material and organic solvent required (Basheer et al., 2010). The successful extraction of patulin from apple juice was achieved by utilizing ethyl acetate, while from whole apples, an ammonium acetate-acetic acid solution in a methanol-water solution (95:5, v/v) was used (Ohta et al., 2017). Vortex-assisted liquid-liquid microextraction (VALLME) is another technique that relies on lLLE. In this method, a small amount of extraction solvent is mixed into an aqueous sample using vortex agitation, creating tiny droplets that enhance the extraction of target compounds by minimizing the diffusion distance and increasing the surface area available for extraction (Yiantzi et al., 2010). After the phases have been separated, the organic layer can be collected and used directly in the chromatography system (Table 4).

5.4 Solid-phase extraction method

Solid-phase extraction (SPE) is another environmentally-friendly approach in chromatographic analysis sample preparation. SPE techniques have become increasingly popular due to their several advantages over the conventional LLE methods, such as the requirement of a reduced number of samples and organic solvents for analysis, elevated recovery rates, and shorter processing durations (Kole et al., 2011). SPE works by dissolving or suspending samples in solvents (such as acetonitrile) and allowing them to pass through a solid phase. SPE works by dissolving or suspending samples in solvents (such as acetonitrile) and allowing them to pass through a solid phase separating analytes according to how well they adhere to the sorbent. This method makes it possible to isolate, concentrate, and purify the target molecule. in this investigation of patulin contamination in fruit-based commodities such as juices, purees, and jams, researchers recognized the value of SPE and implemented it individually (Vandendriessche et al., 2013; Seo et al., 2015; Arghavani-Beydokhti et al., 2018).

TABLE 4 *Alternaria* mycotoxin detection using LC-UV/DAD/FLD/ECD/ELSD.

Alternaria	Sample	Analytical Technique	Clean-Up/Extraction	LODs	References
AOH	Tomato Pulp	HPLC-DAD	Methanol	5.0 µg kg^{-1}	da Motta and Valente Soares, 2001
AME	–	–	–	–	–
TeA	–	–	–	–	–
ATX-I	Tomatoes	HPLC-ECD	Aqueous ammonium sulphate and methanol	Subparts/million	Visconti et al., 1991
ATX-II	–	–	–	–	–
AOH	Tomato Paste	HPLC-FLD SPE	QuEChERS	1.93ug/L^{-1}	Fente et al., 1998
AOH	Pomegranate Fruit and Juice	HPLC-DAD	SPME	15–20 µg kg^{-1}	–
AME	–	–	–	–	–
TeA	–	–	–	–	Myresiotis et al., 2015
TeA	Wine	LC-DAD	SPE	70 µg L^{-}	Abramson et al., 2007; Myresiotis et al., 2015
AOH	Tomato and Products	LC-UV	SPE	5 ug/kg^{-1}	–
AME	–	–	–	10 ug/kg^{-}	–
TeA	–	–	–	20 ug/kg^{-1}	Solfrizzo et al., 2004
AME	Tomato and Products	LC-UV	SPE	3 ug/kg^{-1}	Stack et al., 1985
TeA	–	–	–	20 ug/kg^{-1}	–
AOH	–	–	–	0.8-0.4 ng/ml	–
	–	–	–	0.5-0.4 ng/ml	–
AME	wine and juice	LC-UV	SPE	39 ng/ml	–
	Red grape	LC-UV	SPE	0.27 ng/ml	–
	White wine	–	–	–	Broggi et al., 2013

Nevertheless, as with every other methodology, SPE also has certain boundaries. The utilization of clarified juice, dried fruit, and other fruit-based products with intricate compositions was deemed unsuitable (Ji et al., 2017). SPE columns such as MycoSep 228 are commercially available and have been specifically designed for the detection of patulin in food. In this study, the effectiveness of the MycoSep 228 column was assessed for identifying patulin in homemade apple and hawthorn beverages (Seton et al., 2012). New sorbents have been created as an alternative to commercially available SPE columns to improve the effectiveness of separating patulin from other components of the fruit matrix. The removal and pre-concentration of patulin from fruit products have been studied using a variety of polymers as solid-phase sorbents (Anfossi et al., 2010; Zhou et al., 2019).

5.5 Molecularly imprinted polymers extraction

Molecularly imprinted polymers (MIPs) were created utilizing the oxindole molecule as a template to selectively bind mycotoxins in apple juice (Members et al., 2012). Methacrylic acid, which serves as the functional monomer, and ethylene glycol dimethacrylate were used to create the polymer *via* non-covalent free radical polymerization (cross-linker). Regrettably, the proposed method is overly complicated to prepare MIPs, and the recovery gains are only marginal (84.31 to 88.89%) was observed. Zho and colleagues proposed a radical polymerization method called Grafting as an alternative approach to MIPs (Members et al., 2012; Huen and Daoud, 2017; Zhou et al., 2019), In the production of a patulin-selective SPE sorbent, a silica surface was initially pre-grafted with amino groups, and MIP was created in the presence of acrylamide (functional monomer), ethylene glycol dimethacrylate (cross-linker), and 6-hydroxynicotinic acid (a template substitute) in a series of steps. The ultimate material was successfully employed to analyze patulin, with the analyte recoveries ranging from 90.08 to 96%(Sadok et al., 2019a)

5.6 QuECHERS, and safe extraction method

The QuEChERS approach, which stands for quick, easy, cheap, effective, rugged, and safe, has gained popularity as a pre-treatment method for chromatographic analysis due to its many benefits. This method typically involves the extraction of a 10 g homogenized sample in 10 mL acetonitrile, followed by the addition of a salt mixture (4 g magnesium sulfate anhydrous and 1 g sodium chloride) and separation of the extract. Then, a dispersive solid-phase extraction (DSPE) is performed to purify the 1 mL acetonitrile extract using 150 mg anhydrous magnesium sulfate and 25 mg PSA sorbent (Zhao et al., 2012). Dispersive solid-phase extraction (dSPE) has several advantages over traditional SPE. It eliminates the need for conditioning and elution steps, reduces sorbent consumption, and eliminates the need for additional equipment such as vacuum/pressure or flow control devices. In dSPE cleaning, sorbents are chosen to selectively retain interfering compounds extracted from the sample matrix, while retaining the analytes in the liquid phase. Several dSPE sorbents have been evaluated to ensure satisfactory recoveries and precise results. For example, the PSA sorbent effectively removes various polar matrix components found in food extracts, including organic acids, polar pigments, and sugars. Carbon-based sorbents are suitable for removing carotenoids, chlorophyll, and sterols, while octadecyl silica (C_{18}) is recommended for samples with high fat and wax content (Sugitate et al., 2012). The original QuECHERS procedure has since been modified to suit specific applications better. Instead of acetonitrile, the extraction solvents and salt mixture composition used in the separation and purification steps were optimized for patulin quantification in fruit matrices (Dzuman et al., 2015) used methanol, a water-acetonitrile mixture (Sirotkin and Kuchierskaya, 2017), or acetonitrile that has been acidified with acetic acid (Burin et al., 2011). To extract patulin, it is possible to utilize the aforementioned method. In order to improve pH control and increase recovery rates, it may be necessary to add buffering salts like sodium citrate and sodium hydrogen citrate sesquihydrate to the standard sodium chloride and magnesium sulfate anhydrous mixture. This modification can improve the separation of matrix compounds (Fernandes et al., 2011; Sadok et al., 2018). The purification step was carried out by (Dušek et al., 2018) using dSPE and only in rare cases by traditional SPE columns (Han et al., 2012). Although the QuEChERS methodology offers several benefits, it utilizes a relatively high amount of extraction solvents in comparison to the DLLME and VALLME techniques mentioned earlier.

Furthermore, the type and number of salts used in the salting-out step affect extraction efficiency significantly. To prevent the extraction of unwanted compounds (such as sugars and pigments) from the sample matrix, it is necessary to perform an optimization step to identify the most effective conditions (Abu-Alsoud and Bottaro, 2021). In addition, post-extraction cleaning is typically required with the QuEChERS approach, which can lead to increased preparation time and analysis costs. The multi-step nature of this method can also increase the risk of target compound loss, which can be mitigated by incorporating an internal standard early in the sample preparation process. However, this can introduce another variable that must be accounted for during method development.

5.7 Matrix solid-phase dispersion extraction method

Matrix solid-phase dispersion (MSPD) is an alternative method worth considering for preparing solid and semi-solid samples for chromatographic analysis. This approach involves mechanically mixing the sample with a solid support (typically C_{18} or C_8) to create a single stationary phase, which is then placed into a disposable cartridge. The desired compounds are eluted using an

appropriate solvent, and the resulting eluent can then be further processed (through evaporation, reconstruction, and analysis). However, the MSPD approach can be time-consuming when targeting specific substances, and it requires relatively large amounts of organic solvents for analyte extraction (typically 10-15 mL per sample), as well as careful attention to sample/dispersant mixing and column packing material selection. Before eluting a sample, a column may occasionally need to be washed, as in SPE. This extra step increases the number of organic solvents used and lengthens the sample preparation process (Hasinger et al., 2018). The extraction of patulin from rot apples and apple juice concentrate was tested using the MSPD methodology. The MSPD methodology extracted patulin from rotten apples and apple juice concentrates (Li et al., 2017b).

5.8 Chromatography methods

The most commonly used chromatographic technique for determining patulin in various fruits and their products is HPLC coupled with either ultraviolet (UV) or diode array (DAD) detectors. In HPLC separations, a mobile phase typically consisting of water and a small amount of acetonitrile (5-10%, v/v) is used. To ensure patulin stability in the mobile phase, chloric acid is often added to acidify it, since patulin tends to be more stable in acidic environments (Berthiller et al., 2017; Sadok et al., 2018). This spectrum is used for patulin identification and quantification because the molecule's maximum absorbance is at 276 nm (Piletska et al., 2017; Iqbal et al., 2018; Zhao et al., 2019; Fu et al., 2020). Detection based on this wavelength suffers from low selectivity due to interference from phenolic compounds, especially 5-hydroxymethylfurfural (5-HMF), which is formed during the thermal treatment of food as an intermediate product of acid-catalyzed hexose degradation and the decomposition of 3-deoxyosone in the Maillard reaction (Shapla et al., 2018). Several studies have shown both positive and negative effects of 5-HMF on human and animal health, including its mutagenicity (Perera et al., 2012). 5-HMF is almost undetectable in fresh fruits but abundant in processed foods. Therefore, the presence of 5-HMF may be useful in evaluating thermal damage or aging in food products. Additionally, 5-HMF is the predominant contaminant found in apple-derived samples during mycotoxin analysis (Al-Hazmi, 2010). Both patulin and 5-HMF absorb UV light strongly, have similar retention times under chromatographic conditions, and thus tend to peak overlap. Many HPLC-UV studies took on the analytical challenge of improving patulin and 5-HMF peak separation. Optimal analytical conditions for the analysis of apples and apple-based products have been established (Li et al., 2017c).

Liquid chromatography-mass spectrometry (LC-MS) is a popular tool for food analysis because of its adaptability, specificity, and selectivity. Most studies that focus on patulin monitoring in fruits use LC coupled with triple tandem quadrupole mass spectrometry (LC-QQQ) in negative-ion mode. The patulin precursor ion is typically chosen as m/z 153, which corresponds to a patulin molecule after losing a proton [M-H]. The mobile phase is composed of acetic acid and methanol or acetonitrile (Madani-Tonekaboni et al., 2015). Alternatively, this mycotoxin analysis employs ammonium acetate (Panwalkar et al., 2017; Rodriguez et al., 2021). The target molecule is highly polar, which results in low sensitivity for the LC-MS analysis. The problems can be attributed to the inadequate ionization of patulin under source and atmospheric pressure chemical ionization (APCI) conditions (Nielsen and Larsen, 2015). LC-MS analysis of complex matrices like fruit samples must consider matrix effects that can lead to signal suppression or enhancement of the target molecule. To reduce these effects when quantifying patulin, using an isotopically labeled patulin standard and a matrix-matched methodology is recommended. However, most LC-MS protocols are designed for multi-target analyses, which can reduce sensitivity and may not meet the EU's legal restrictions for patulin quantification in baby food. For example, LC-QQQ has been used to identify 33 pesticides or degradation products in addition to patulin in apples, and the method was validated using four food matrices. In the apple matrix, the LOQ for patulin was 119.7 μg kg-1, with a 77% recovery rate. Patulin had a LOQs of 5 μg L-1 and a 78% recovery rate (Nakatsuji et al., 2015). The patulin levels in various fruit juices (apple, grape, orange, blueberry, lemon, pear, mango, and coconut) and dried fruits (apple, apricot, kiwi, prune, pineapple, papaya, mango, fig) were assessed, with estimated recoveries exceeding 92% for the apple juice matrix. Moreover, utilizing the QuEChERS method for sample preparation of apple and pear-based products prior to LC-MS/MS analysis resulted in higher recoveries ranging from 94% to 104%, with LOQs of up to 10 μg/L for all matrices tested (El-Ramady et al., 2015). The advanced LC method with a triple quadrupole analyzer was used to determine individual patulin levels in four different apple matrices, including juice, fruit, puree, and compote (El-Ramady et al., 2015). The sentence describes the results of a study that used LC-MS/MS method to measure patulin levels in various fresh fruits and their products. The study found that LOQs for all matrices ranged from 2 to 15 μg kg-1, and the average recoveries ranged from 71 to 108 per cent. The fruits and products tested included apples, pears, apricots, peaches, bananas, grapes, plums, strawberries, raspberries, blueberries, blackberries, sour cherries, as well as their juices and pulps. The study aimed to track the levels of patulin in these fruits and products to ensure food safety (Azari et al., 2020). The method using LC-MS/MS has been able to detect patulin levels with LOQs ranging from 0.8 to 2.4 μg kg-1, and it has been applied to the analysis of patulin content in apples and apple-based products. However, it should be noted that this method may not be suitable for the analysis of patulin in complex matrices such as dried or pigmented fruits (such as berries and sour cherries) and their derived products, where LLE-based methods may not be effective It requires changing current analytical protocols or creating new ones, such as more complex sample preparation. Patulin quantification is challenging at the legislative level due to various sample components that adversely affect chromatographic peak resolution and ionization efficiency and lead to unsatisfactory recovery. Recent advances in this field have primarily focused on SPE and QuEChERS adjustment. It enabled the development of efficient protocols for removing interfering compounds (such as phenolic molecules) from complex fruit matrices. Several

modifications to the original QuEChERS protocol, including using MIPs as SPE sorbents, have been proposed to enhance sample cleaning and patulin pre-concentration. Although it is still preferred, the HPLC-UV method has a lengthy analysis time for mycotoxin analysis in fruit commodities. (This is required for good peak resolution) and, as a result, relatively high mobile phase consumption. Because of this, mass spectrometry detection and UHPLC systems have surpassed the HPLC-UV method in analyzing organic compounds, which is now being phased out. Because of poor ionization in a mass spectrometer source, patulin is frequently omitted from a list of multi-target mycotoxin analyses. Instead, it is determined on an individual basis through a separate protocol. It appears to be a significant barrier to developing multi-mycotoxin approaches, so resolving the patulin sensitivity issue on the future path of LC-MS-based methods will require careful attention.

Tin layer chromatography has been employed as a rapid and direct qualitative analysis technique in various fields, including the determination of *Alternaria* mycotoxin levels. In a study using a solvent system of chloroform/acetone (97:3, v: v) and TLC-UV, *Alternaria* mycotoxins (AME, AOH, TeA, ATX-I, and ATX-II) were detected in tomatoes. The predominant mycotoxins found in spoiled tomatoes were AOH, AME, and TeA, with LODs of 100, 100, and 700 μg kg^{-1} for AOH, AME, and TeA, respectively (Gašparović et al., 2015). The presence of AOH, AME, ALT, ATX-I, and TEN in *A. alternata* IMI 354942 cultures was detected using TLC-UV. The LODs for AOH, AME, ALT, ATX-I, and TEN were 250, 125, 250, and 250 μg L^{-1}, respectively. Compared to high-performance thin-layer chromatography (HPTLC), TLC showed superior separation efficiency and detection sensitivity (Gökbulut, 2021). The study detected AOH and AME mycotoxins in raspberry, tomato, wheat, and oat samples using HPTLC and densitometry techniques, with a LODs of approximately 60 μg kg^{-1}. The same HPTLC method was also used to quantify AOH, AME, ALT, and TeA mycotoxins in fresh grape juice, must, and wine, with LOQs of 1.5 μg L^{-1} for AOH and AME, and 7.5 μg L^{-1} for TeA (Liang et al., 2021). Although TLC has lower separation efficiency and detection sensitivity compared to HPLC and GC, it is still an essential tool for mycotoxin detection in various matrices due to several advantages, such as ease of operation, rapidity, cost-effectiveness, and the use of less solvent than LC. Additionally, TLC has no memory effects, making it a more environmentally friendly optio" (Zhang et al., 2021). Furthermore, the detection sensitivity of HPTLC is higher than that of GC and HPLC with a densitometric detector.

In recent years, reversed-phase LC coupled with various classical detectors such as ultraviolet detector (UVD), diode-array detector (DAD), fluorescence detector (FLD), electrochemical detector (ECD), evaporative light-scattering detector (ELSD), and mass spectrometry (MS) has become the preferred method for detecting *Alternaria* mycotoxins, supplanting GC and TLC. Among these detectors, LC-UV is a popular method due to the ability of most organic and inorganic molecules to absorb ultraviolet light. To detect *Alternaria* mycotoxins in carrots, researchers utilized SPE as a pre-treatment step and employed reversed-phase LC with a UV diode array detector (LC-UV/DAD). The study found that LODs for TeA, ATX-I, AME, and AOH were 20, 20, 10, and 5 μg kg^{-1}, respectively (Gotthardt et al., 2019). Canadian ice wines contain TeA, while Estonian grain wines contain LC-DAD AOH (Asam et al., 2012). The LODs for the analytes were determined using LC-DAD, and were found to be 100 μg kg^{-1} and 70 μg L^{-1} for one or more of the *Alternaria* mycotoxins. In the case of TeA, it is known to act as an effective chelating agent, forming complexes with metal ions. Therefore, when detecting TeA, zinc sulfate ($ZnSO_4$) is often added to the mobile phase to enhance the sensitivity of the detection method (Santos et al., 2012), Due to its low sensitivity, LC-DAD is less frequently used than UV detectors to detect *Alternaria* mycotoxins. Contrarily, the LC-UV/DAD method of diode array UV detection has a high sensitivity (De Vargas et al., 2015). A new sampling technique for detecting pesticides in fruits and vegetables has been developed, which is quick, simple, and efficient. QuEChERS, an acronym for quick, easy, cheap, effective, rugged, and safe, is an additional method to SPE and SPME techniques (Lingwal et al., 2022). By employing HPLC-DAD in combination with the QuEChERS extraction method, the levels of AOH, AME, and TEN were measured in both pomegranate fruit and juice, with LODs falling between 15 to 20 μg kg^{-1}, and the LOQs ranging from 50 to 66 μg kg^{-1} (Petsas and Vagi, 2020). HPLC-FLD is an abbreviation for the FLD acronym, which is frequently used in high-performance liquid chromatography. Compared to UV and DAD detectors, it offers greater selectivity and sensitivity, with a LOD of up to μg L^{-1}. The quantity of AOH present in tomato paste was evaluated using HPLC-FLD, following extraction of the sample through SPE cartridges. The LOD of AOH was found to be 1.93 μg L^{-1}, with a linear range of 5.2-196 μg L^{-1} (Zhang et al., 2020a). Due to the absence of fluorescent functional groups in TeA, its detection is not feasible with the FLD detector. Consequently, LC-UV and LC-DAD methods are more commonly employed than LC-FLD for the identification of *Alternaria* mycotoxins, as they can detect TeA as well. LC-ECD, a popular technique for analyzing trace samples, has a sensitivity of 106 μg L^{-1}, and is exclusively used to detect electroactive molecules that can be readily oxidized or reduced. AOH, AME, ATX-I, and ATX-II, all being electroactive, are among the *Alternaria* mycotoxins that can be detected using LC-ECD (Man et al., 2017). To enhance the detection sensitivity of ATX, a dual in-series electrode system can be coupled with an HPLC method in the "redox" mode (Sontag et al., 2019). Samples containing ATX-I and ATX-II were extracted from maize, rice, and tomatoes that were infected, and LOD levels achieved were below one part per million (sub-ppm) (Sontag et al., 2019). The detection of AAL toxin has traditionally depended on time-consuming derivatization or immunoassay methods since it lacks a UV chromophore, which is why ELSD-LC has been used as an alternative (Lagarde et al., 2018). A sensitive and rapid analytical method was developed for the quantitative detection of AAL toxins in fungal culture, which involves coupling a C_{18} reverse phase HPLC to an ELSD, with a LOD of around 6000 μg L^{-1}. However, this ELSD method emits harmful exhaust gas during the detection process and requires a signal transducer in conjunction with LC and high-pressure nitrogen or air configuration. In recent years, MS detection techniques, especially LC-MS/MS or LC-MSN interfaces

based on ESI or APCI, have outperformed FLD, UV, DAD, and ECD detectors in the simultaneous detection and quantification of *Alternaria* mycotoxins in various samples without derivatization. The ESI source has been the most commonly used for detecting *Alternaria* mycotoxins due to its higher sensitivity than APCI. Using LC-MS analytical methods, dibenzopyrone derivatives Toxins AOH, AME, and ALT can be detected. A pretreatment method utilizing SPE was employed for the detection of AOH and AME in flavedo, using HPLC-MS/MS. The results indicated a linear range of 0.50 -20.0 μg kg^{-1}, with a LOD and LOQ lower than 0.13 and 0.50 μg kg^{-1}, correspondingly, and an RSD of 114.4%. Subsequently, a HPLC-MS/MS method was developed to detect 23 mycotoxins in 6 food supplements (Myresiotis et al., 2015). The sample was initially obtained using a mixture of ethyl acetate and formic acid (95:5 v/v), followed by purification using an OASIS HLBTM SPE column. of the 23 mycotoxins, the LODs for ALT, AOH, and AME were 2, 8, and 30 μg kg^{-1}, respectively. The LOQs for these mycotoxins were approximately three times higher than their respective LODs. Furthermore, although the sample preparation differed, HPLC-MS/MS detected 23 mycotoxins in sweet pepper (Rico-Yuste et al., 2018). The multi-mycotoxin LC-MS/MS method that was developed successfully fulfilled the method performance criteria that were specified in Commission Regulation (EC) no. 401/2006 (Matumba et al., 2015). The QuEChERS extraction method can be employed in conjunction with LC-DAD and LC-MS techniques to simultaneously detect *Alternaria* mycotoxins. AOH and AME, both of which are multi-mycotoxins, were extracted utilizing QuEChERS and subsequently identified through LC-ESI-MS/MS. The LODs for AOH and AME were 10 and 6g kg^{-1}, respectively (Zervou et al., 2017). UHPLC (ultra-high-performance liquid chromatography), has been extensively utilized in the analysis of mycotoxins, as well as in the LC and HPLC methods mentioned earlier. For example, the QuEChERS method was utilized to extract AOH, AME, TEN, ALT, and ATX-I from tomato products, fruit juices, and vegetable juices, with detection being carried out through UPLC-MS/MS. The findings indicated that LODs, LOQs, and LOQs of these toxins in tomato products, fruit juices, and juices ranged from 3.0-8.3, 9.8-61.5, and 1.1-5.7 μg kg^{-1}, respectively (Smoluch et al., 2016). Regenerate Performed detection of *Alternaria* mycotoxins with different chemical structures at the same time successfully used HPLC-ESI-MS/MS in foods from German markets to quantify the nine *Alternaria* toxins (AOH, AME, TeA, ALT, ALT, TEN, and ATX-I). The LODs and LOQs were determined to be 2.8-5.4 and 9.3-8 μg kg^{-1}, respectively, while for TA 2, the values were found to be 1.2-7 and 3.8-55 μg kg^{-1}. LC-APCI-MS/MS was used to identify five *Alternaria* mycotoxins, including AOH, AME, TeA, ALT, and TEN, in various food products such as apple juice, beers, tomato products, olives, and dried basil, with LODs and LOQs ranging from 0.16-2.31 μg kg^{-1} and 0.54-41.04 μg kg^{-1}. AOH was found to be the most common *Alternaria* mycotoxin, followed by ALT. Utilized UPLC-ESI-MS/MS to quantify the levels of these mycotoxins in tomato products, and TeA was found to be the most frequently detected toxin, with a maximum concentration of 790 μg kg^{-1} in 81 out of 84 samples (Lawal et al., 2019). On the other hand, AOH and AME were found to have lower concentrations, ranging from 1 to 34 μg kg^{-1} and 5 to 9 μg kg^{-1}, respectively. Therefore, the results suggest that TeA is the most common *Alternaria* mycotoxin (Noser et al., 2011). LC-ESI-MS/MS analysis detected AOH, AME, ATX-I, and TEN mycotoxins in various samples, in addition to other mycotoxins (Puntscher et al., 2018; Gotthardt et al., 2019). (Tables 5 , 6).

5.9 Gas chromatography method

Alternaria mycotoxins were also used with various detection techniques to detect GC has high sensitivity and selectivity, especially GC-MS, and can detect some substances in the blend. Despite being small, non-volatile, and polar, many *Alternaria* mycotoxins can still be detected using methods that are sensitive to non- and half-polar, volatile, and semi-volatile compounds (Anfossi et al., 2016). Thus *Alternaria* mycotoxins usually need to be derived before the analysis of GC-MS (Anfossi et al., 2016). TeA was derivatized by a compound of acetyl-trimethyl silane (6:2:9, v: v:v) and a mixture of acetylated ionization silane, trimethylated silane, and pyridine; TeA's LOD was 100 μg kg^{-1}, using GC and fire ionization sensor.

The researchers employed a two-step process of derivatization followed by GC-MS analysis using heptafluorobutyrate (HFB) and Trimethylsilyl (TMS) to detect mycotoxins such as AOH, AME, ALT, ALTX-I, and TeA (Amatatongchai et al., 2019). The results showed that GC separation of the *Alternaria* mycotoxin before MS detection is sufficient for both the HFB and TMS derivatives. The results showed that GC separation of the *Alternaria* mycotoxin before MS detection is sufficient for both the HFB and TMS derivatives. Apple juice contained AOH and AME LODs of 1 μg kg^{-1}. Despite their high sensitivity, the GC-MS methods described above have not been widely used for detecting *Alternaria* mycotoxins. The main reason for this is that most *Alternaria* mycotoxins samples require derivatization in GC-MS detection process has certain limitations such as matrix interference, inadequate repeatability, high time consumption, costly derivatization reagents, and complicated operational procedures. Moreover, the technique can experience memory effects due to earlier sample injections. As a result of the time-consuming derivatization reactions required, the use of GC to determine *Alternaria* mycotoxins is limited.

5.10 SIDA method

Quantitative results must be corrected using special methods and suitable internal standards to consider the significant ion suppression that LC-MS experiences (Al-Lami et al, 2019). Several sample preparation and separation techniques are required because *Alternaria* can infest various analytical materials. SIDA (stable isotops dilution assays) is a fantastic method for recouping analyte losses during sample preparation and minimizing ion suppression at the ESI interface (Pavicich et al.,

TABLE 5 *Alternaria* mycotoxin detection using LC–MS.

Alternaria	Sample	Analytical Technique	Extraction/Clean-Up	Limit of Detection (LODs) (μg/kg)	(LOQs : Limit of quantitation) (μg/kg)	References
AOH	Wines, grapes juices	LC-ESI-MS/MS	SPE	0.01-0.8	–	–
AME	and cranberry juice	–	–	–	–	Juan et al., 2017
AOH	Tomato, wine, apple	LC-ES-MS/MS	Water-formic acid acetonitrile	–	1.5-5.0	–
AME	apple juices	–	(84/16/1, v/v/v)	–	–	–
ALT	–	–	–	–	–	López et al., 2016
AOH	FRUITS	UPLC/ESI-MS/MS	Acetonitrile/water/acetic 79:20:1, v/v/v	2	–	–
AME	–	–	–	0.1	–	–
ALT	–	–	–	6	–	–
TEN	–	–	–	0.5	–	Haque et al., 2020
AOH	Beer	UHPLC-orbitrap MS	Acetonitrile	–	–	–
AME	–	–	–	–	–	–
ALT	–	–	–	–	–	Tittlemier et al., 2019
AOH	tomato products	UPLC–MS/MS	QuEChERS	3.1-18.3	9.8-61.5	–
AME	Fruits	–	–	–	1.1-5.7	Walravens et al., 2016
AOH	fruits such as apple	SPE	–	–	44563	–
AME	orange, sweet cherry	–	–	–	–	–
ALT	and tomato	–	–	–	–	Wang et al., 2021
AOH	tomato products	UPLC-ESI-MS/MS	SPE	4	–	–
AME	–	–	–	1	–	–
ALT	–	–	–	2	–	–
TeA	–	–	–	2	–	–
ATX-1	–	–	–	–	–	–
TEN	–	–	–	2	–	Noser et al., 2011
AOH	dried fruits	LC–MS/MS ESI	Acetonitrile/water/acetic acid, 79:20:1, v/v/v	5	–	–
AME	–	–	–	8	–	–
ATX-1	–	–	–	3	–	–
TEN	–	–	–	0.4	–	Warth et al., 2012
AOH	apple Juices, beers, tomatoes, olives	LC-APCI-MS/M	SPE	0.16-12.31	0.54-41.04	Garganese et al., 2016
AOH	tomato and fruit juices	HPLC-ESI-MS/MS	Methanol/water/formic acid (49:50:1, v/v/v)	0.04–0.4	0.6–9.3	–
AME	–	–	–	0.8–24 2.5–81	0.1–1.2	–

(Continued)

TABLE 5 Continued

Alternaria	Sample	Analytical Technique	Extraction/Clean-Up	Limit of Detection (LODs) (µg/kg)	(LOQs : Limit of quantitation) (µg/kg)	References
ALT	–	–	–	1.3–19	4.4–62	Hickert et al., 2016
TeA	–	–	–	–	–	–
TeA	Olive, olive oil, olive husks	–	–	–	100 µg kg^{-1}	Nunes et al., 2011
ATX-1	–	–	–	–	200 µg kg^{-1}	–
ALT	–	–	–	–	100 µg kg^{-1}	–
AME	–	–	–	–	30 µg kg^{-1}	–
AOH	–	–	–	–	50µg kg^{-1}	–
AOH	tomatoes	TLC-UV	–	–	100 µg kg^{-1}	–
AME	–	–	–	–	100 µg kg^{-1}	–
TeA	–	–	–	–	700 µg kg^{-1}	–
AOH	Fruit and vegetable Products	TLC-UV	–	–	3 µg kg^{-1}	Gagic et al., 2018
AOH	Raspberries, tomatoes	HPTLC-densitometry	–	–	–	–
AOH	Grape juice, and wine	HPTLC	–	–	60 µg kg^{-1}	Matysik and Giryn, 1996
AME	–	–	–	–	1.5 µg L^{-1}	
TeA	–	–	–	–	7.5 µg L^{-1}	Berthiller et al., 2017

2020). Furthermore, SIDA is a useful tool in analytical applications, such as trace analysis, increasing the accuracy of quantitative results and improving the specificity of the determination (Graybill and Bailey, 2016). Thus far, the primary applications of SIDA for mycotoxin analysis have received critical attention (Malachová et al., 2018). HPLC-MS/MS was used to detect AOH and AME in fruit juices, and the reproducibility of the method was tested using spiked apple juice. The results showed that the reproducibility for AOH and AME was 100.5 ± 3.4% and 107.3 ± 1.6%, respectively. The LODs for AOH and AME were 0.03 and 0.01 µg/kg, and the LOQs were 0.09 and 0.03 µg/kg, respectively (Asam and Rychlik, 2015). Internal standards include [$^{13}C_{20}$]-ATXs, [$^{13}C_{15}$] AOH, and

TABLE 6 *Alternaria* mycotoxin detection using LC-MS and SIDA.

Mycotoxin	Sample	Analytical Techniques	Clean-up/extraction	LOQs	LODs	Reference
TEN	Juice	SIDA, LC-MS/MS	C_{18}-phenyl SPE	–	0.10-0.99 ug/kg^{-1}	Liu and Rychlik, 2013
TeA	Tomato	HPLC-MS/MS, SIDA	RP18-SPE	2.89 µg kg^{-1}	0.86 µg kg^{-1}	Lohrey et al., 2013
TeA	Beverages	SIDA, LC-MS/MS	C_{18}-SPE	–	–	Asam and Rychlik, 2013
TeA	fruit juices	SIDA, LC-MS/MS	C_{18}-SPE	0.5 µg kg^{-0}	0.15 µg kg^{-1}	Berthiller et al., 2018
TeA	Tomato	SIDA, LC-MS/MS	–	0.3 µg kg^{-1}	0.1 µg kg^{-1}	Asam et al., 2011
AME	fruit products	–	–	0.1–5 µg kg^{-1}	–	Asam and Rychlik, 2015
AOH	fruits,	SIDA, HPLC-MS/MS	RP18-SPE	–	13–250 µg kg^{-1}	Ma et al., 2019
AOH	juice	SIDA, HPLC-MS/MS	RP18-SPE	0.09 µg kg^{-1}	0.03 µg kg^{-1}	Man et al., 2017
AME	Apple Juice	UPLC-MS/MS	C_{18}	–	2.20–3.10ug/L	–
TeA	Apple Juice	UPLC-MS/MS	C_{18}	–	11.90–20.60	–
AME	Apple Jam	UPLC-MS/MS	C_{17}	–	3.36-4.42ug/kg^{-1}	–
	Apple Vinegar	UPLC-MS/MS	C_{18}	–	15.50 ug/kg^{-1}	Quan et al., 2020

[$^{13}C_{14}$]-AME. In a stable isotope dilution, alternative labeling of [$^{13}C_{20}$]-ATXs, [$^{13}C_{14}$]-AOH, and [$^{13}C_{15}$]-AME were used as internal standards. The study found that samples contaminated with ATX may also contain other *Alternaria* toxins like AOH, AME, and TEN, but not necessarily in the same sequence(Liu and Rychlik, 2015). Additionally, the recoveries showed a range of 96 to 109%, while the inter- and intra-day RSDs were below 13%. Furthermore, the LODs and LOQs for AOH, AME, ATX-I, and ATX-II were determined to be 0.36 and 1.1 μg kg^{-1}, 0.09 and 0.27 μg kg^{-1}, 0.36 and 1.1 μg kg^{-1}, and 0.53 and 1.6 μg kg^{-1}, respectively [279, 280] is an internal standard. After acetoacetylation with diketene, the stable-isotope-labeled [$^{13}C_6$ ^{15}N] To quantify TeA in tomatoes using LC-MS/MS, [$^{13}C_6$, ^{15}N] TeA was synthesized by Dieckmann intramolecular cyclization and used as an internal standard. The LOD and LOQ for TeA were determined to be 0.1μg kg^{-1} and 0.3 μg kg^{-1}, respectively. The TeA content in various foods was determined using SIDA, with LODs of 0.15 μg $kg^{-1}$for fruit juices, 1.0 g kg^{-1} for cereals, and 17 μg kg^{-1} for spices (Pulkrabová et al., 2013). The study also investigated the TeA levels in infant foods and beverages. Popkin reported that the median TeA content in infant tea infusions was 2 μg L^{-1}, while fennel tea infusions had TeA levels as high as 20 μg L^{-1}. Moreover, the average TeA content in pureed food was found to be higher for tomatoes at 25 μg kg^{-1}, and for bananas and cherries at 80 μg kg^{-1} (Asam and Rychlik, 2013) (Table 7).

5.11 Enzyme-linked immunosorbent assay method

High-end equipment and highly trained personnel are required for instrumental analytical methods such as GC-MS and LC-MS. Despite the limitations of instrumental analytical methods, enzyme-linked immunosorbent assay (ELISA) has become a popular research focus for quantitative and semi-quantitative mycotoxin detection, including *Alternaria* toxin. ELISA stands out due to its simplicity, miniaturization, speed, and portability. It has been used to detect AAL, AOH, and TeA, and can be applied to determine the presence of *Alternaria* mycotoxin in foods. TeA was derivatized with succinic anhydride and then coupled with KLH to screen polyclonal antibodies. Competitive ELISA developed using these polyclonal antibodies showed higher sensitivity for TeA acetate than for TeA, with an average standard curve detection limit of 5.4 ± 2.0 μg L^{-1} for TeA acetate. The LODs for TeA in apples and tomatoes was found to be 25-50 μg kg^{-1}(Gross et al., 2011; Wang et al., 2020).

6 Management of patulin and *Alternaria* mycotoxins in fruits

It is necessary to manage the presence of Patulin and *Alternaria* mycotoxins in fruits because these toxins can pose significant health risks to humans if consumed in large quantities. Patulin is a toxic secondary metabolite produced by several fungal species, including *Penicillium*, *Aspergillus*, and *Byssochlamys*, commonly found on fruits such as apples, pears, and grapes. Patulin has been linked to various health problems such as acute gastrointestinal distress, nausea, vomiting, and immune system suppression in animal studies, and possibly carcinogenicity in humans (Azam et al., 2021). On the other hand, *Alternaria* mycotoxins, including AOH, AME, and tentoxin, are also commonly found in fruits such as tomatoes, strawberries, and apples, and have been associated with several health problems such as genotoxicity, immunotoxicity, and carcinogenicity in animal studies, although their effect on humans is still unclear. Therefore, it is important to manage the levels of patulin and *Alternaria* mycotoxins in fruits to ensure that the fruits consumed by humans are safe and do not pose any health risks (El-Kady and Abdel-Wahhab, 2018). The management of patulin and *Alternaria* mycotoxins in fruits can be achieved through a combination of good agricultural practices, post-harvest handling, and processing to minimize its presence in fruits. The potential measures are pre-harvest management including proper crop management, including irrigation, fertilization, and pest control, can help reduce the occurrence of fungal infections in fruits. Use of resistant cultivars can also help reduce infection rates. Harvest management including proper harvesting techniques, including the use of sanitized equipment and the removal of infected fruits, can help minimize fungal contamination. Post-harvest handling including proper storage and transportation practices, temperature control and regular cleaning and sanitization, can help prevent fungal growth and mycotoxin production (Nan et al., 2022). Processing appropriate processing techniques, including washing, sorting, and disinfection, can help reduce the levels of mycotoxins in fruits and fruit products. And monitoring including regular monitoring of fruit and fruit products for mycotoxin levels can help identify potential contamination and allow for corrective action to be taken (Saleh and Goktepe, 2019). Certain management strategies including, physical methods, chemical methods, and biological method, to reduce the amount of patulin and *Alternaria* mycotoxins contamination in fruits are discussed below.

6.1 Physical methods

There are several physical methods that can be used to reduce the levels of patulin and *Alternaria* mycotoxins in fruits and their derived products. The most considerable physical methods to reduce these mycotoxins include; Thermal treatment (also known as heat treatment is one of the most common methods used to reduce mycotoxin levels in food products. High temperatures can denature or destroy the mycotoxins, but this method may also result in the loss of some nutrients and changes in the organoleptic properties of the food product) (Shanakhat et al., 2018); Irradiation (Irradiation is another method that can be used to reduce mycotoxin levels in food products. It involves exposing the food product to high-energy radiation, such as gamma rays, which can break down the mycotoxins. This method is effective in

TABLE 7 Country wise regulations on Maximum permitted limits (MPLs) of patulin and *Alternaria* content in fruits and their derived products.

Country	Commodity	Maximum Permitted Limits (MPLs)	References
China	Fruit products (with the exception of hawthorn sheet) Fruit and vegetable juice Liquor	50 μg kg^{-1}	Li et al., 2017a
United States of America	Apple juice, apple juice concentrate, and apple juice component of a food that contains apple juice as an ingredient	50 μg kg^{-1}	Cai et al., 2020
MERCOSUR member states	Fruit juice	50 μg kg^{-1}	
Cuba	Fruits	50 μg kg^{-1}	
European Union member states	Fruit juices, concentrated fruit juices as reconstituted and fruit nectars,	50 μg kg^{-1}	Van Egmond and Jonker, 2008
	Spirit drinks, cider, and other fermented derived from apples or containing apple juice	50 μg kg^{-1}	
	Solid apple products, including apple compote, apple puree intended for direct consumption (except baby foods)	25 μg kg^{-1}	
	Apple juice and solid apple products, including apple compote and apple puree, for infants and young children	10 μg kg^{-1}	
	Baby foods other than processed cereal-based foods for infants and young children	10 μg kg^{-1}	
Iran	Fruit juices, nectarine, and fruit drinks	50 μg kg^{-1}	Nascimento and Taniwaki, 2023
Israel	Apple juice	50 μg kg^{-1}	
Japan	Apple juice	50 μg kg^{-1}	
Republic of Korea	Apple juice, apple juice concentrate	50 μg kg^{-1}	
Republic of Moldova	Juices, canned vegetables, fruits	50 μg kg^{-1}	
Morocco	Apple juice (products)	50 μg kg^{-1}	
Russian Federation	Apple, tomato, sea-buckthorn (canned)	50 μg kg^{-1}	Dankvert, 2010
	Alcoholic free beverages, including juice containing and artificially mineralized drinks	50 μg kg^{-1}	
	Vegetable, fruit juices, beverages, concentrates	50 μg kg^{-1}	
	Semi-finished products (tomato pulp, apple pulp)	50 μg kg^{-1}	
	James, marmalade, fruit pastes, comfitures, fruit and berries crushed with sugar and other fruit and berry concentrates with sugard	50 μg kg^{-1}	
Singapore	Fruit Juices	50 μg kg^{-1}	Lee et al., 2018
	Food for infants or young children (except processed cereal-based foods)	10 μg kg^{-1}	
	Food containing fruit juice as ingredient	50 μg kg^{-1}	
South Africa	All foodstuffs	50 μg kg^{-1}	Van Egmond and Jonker, 2008
Switzerland	Fruit juices	50 μg kg^{-1}	
Armenia	Tomato paste, apple	50μg kg^{-1}	
Belarus	Mushrooms, fruits, vegetables	50 μg kg^{-1}	
Ukraine	Vegetable and fruit-berry preserves and mixes for baby food.	20 μg kg^{-1}	
	Vegetables, including potatoes, fruit and grapes, berries; vegetable, fruit, berry preserves in cans and jars	50 μg kg^{-1}	
Canada	Fruits and fruit juices	50 μg kg^{-1}	Canada, 2020
Australia New Zealand	Fruits, Apple juices, berries	50 μg kg^{-1}	Sirisena et al., 2015

(Continued)

TABLE 7 Continued

Country	Commodity	Maximum Permitted Limits (MPLs)	References
Brazil	Fruit and their products	50 μg kg^{-1}	Brazil, 2001
India	Apple juices	50 μg kg^{-1}	Shukla et al., 2014
Mexico	Fruit and juices	50 μg kg^{-1}	Shukla et al., 2014
European Union	Tomato juices	30 μg/kg	C.R.N, 2006; Van Egmond and Jonker, 2008
Japan	Apple juices	30 μg/kg	Li and Beghin, 2014
China	Apple and tomato juices	50 μg/kg	

reducing mycotoxin levels but may also affect the sensory properties of the fruits and their derived products) (Akhila et al., 2021); Ultrasonic treatment (It is a non-thermal physical method that can be used to reduce mycotoxin levels in food products. This method involves exposing the food product to high-frequency sound waves, which can disrupt the mycotoxins' structure and reduce their toxicity (Sipos et al., 2021). This method is effective in reducing mycotoxin levels while preserving the nutritional value and sensory properties of the food product); Cold plasma treatment (another non-thermal physical method that can be used to reduce mycotoxin levels in food products. This method involves exposing the food product to low-temperature plasma, which can break down the mycotoxins. This method is effective in reducing mycotoxin levels but may also affect the sensory properties of the food product) (Misra et al., 2019); and High-pressure processing (HPP) (HPP is a method that involves subjecting the food product to high pressure, which can destroy the mycotoxins. This method is effective in reducing mycotoxin levels while preserving the nutritional value and sensory properties of the food product) (Woldemariam and Emire, 2019). In conclusion, there are several physical methods that can be used to reduce mycotoxin levels in fruits and their derived products. These methods are effective in reducing mycotoxin levels while preserving the nutritional value and sensory properties of the food product. However, the choice of the most appropriate method will depend on the type of food product, the nature of the mycotoxin, and the desired level of reduction.

It worth to mention that, in the physical methods for the detection of *Alternaria* and patulin in fruits, high temperature treatments have been shown to be effective in controlling fungal growth and mycotoxin production. However, it can also cause damage to the fruit, reducing its quality and shelf life (Pal et al, 2017). Similarly, UV-C radiation has been reported to be effective in reducing *Alternaria* and patulin in fruits. However, it can also damage the fruit surface and lead to changes in color and texture. In contrast, cold plasma treatment has been shown to be effective in reducing *Alternaria* and patulin in fruits without causing any damage to the fruit surface. As it is a non-thermal process and does not affect the fruit quality and nutritional value (Khan et al., 2019). Moreover, MAP is also an effective method for controlling fungal growth and mycotoxin production in fruits. It involves modifying the atmosphere inside the package to slow down the ripening process and inhibit fungal growth. It is a non-invasive method and does not affect the fruit quality and nutritional value.

6.2 Chemical methods

There are several chemical methods that have been developed for patulin and *Alternaria* mycotoxin reduction in fruits. Some of the most commonly used ones include; Sodium bisulfite treatment (Sodium bisulfite is a chemical that can reduce the levels of patulin and *Alternaria* mycotoxins in fruits. The treatment involves soaking the fruits in a solution of sodium bisulfite for a specific period of time, which can vary depending on the type of fruit and the concentration of the solution); Hydrogen peroxide treatment (Hydrogen peroxide is a strong oxidizing agent that can be used to reduce the levels of patulin and *Alternaria* mycotoxins in fruits. The treatment involves spraying the fruits with a solution of hydrogen peroxide, which can effectively degrade the mycotoxins) (Paster and Barkai-Golan, 2008); Sodium hypochlorite treatment (Sodium hypochlorite is a chemical that is commonly used as a disinfectant. It can also be used to reduce the levels of patulin and *Alternaria* mycotoxins in fruits. The treatment involves washing the fruits with a solution of sodium hypochlorite, which can effectively remove the mycotoxins (Karlovsky et al., 2016b); Ozone treatment (Ozone is a powerful oxidizing agent that can be used to reduce the levels of patulin and *Alternaria* mycotoxins in fruits. The treatment involves exposing the fruits to ozone gas, which can effectively degrade the mycotoxins (Afsah-Hejri et al, 2020); and Ultraviolet treatment (Ultraviolet light can be used to reduce the levels of patulin and *Alternaria* mycotoxins in fruits. The treatment involves exposing the fruits to ultraviolet light for a specific period of time, which can effectively degrade the mycotoxins). It is important to note that while chemical methods can be effective in reducing the levels of mycotoxins in fruits, they can also have negative effects on the quality and nutritional value of the fruits (Akhila et al., 2021). Therefore, it is important to carefully consider the potential risks and benefits of using these methods before implementing them.

It is noteworthy that, in the chemical methods for the detection of *Alternaria* and patulin in fruits, these chemical methods require specialized equipment and trained personnel, making them more costly and time-consuming than other methods such as visual inspection. False positives and false negatives can also occur if the

sample is not properly prepared or the method is not sensitive enough to detect low levels of contamination. In contrast, these chemical methods are highly sensitive and specific, allowing for accurate detection and quantification of *Alternaria* and patulin in fruit samples (Wang et al., 2016). They are also able to detect low levels of contamination, which is important for ensuring the safety of fruit products for consumption. These methods have been validated and widely used in food safety research and regulatory compliance programs.

6.3 Biological method

To reduce the risk of exposure to patulin and *Alternaria* mycotoxin, various biological methods have been developed for these mycotoxin reduction in fruits and their derived products. Some of the established biological methods include; Biocontrol agents (certain microorganisms such as bacteria, yeast, and fungi can inhibit the growth of patulin and *Alternaria* producing fungi by producing certain antagonistic compounds. For instance, the use of bacteria such as Bacillus subtilis and Pseudomonas fluorescens has been found to be effective in reducing patulin levels in apples (Narayanasamy and Narayanasamy, 2013). Similarly, the application of yeast such as Metschnikowia pulcherrima can reduce *Alternaria* mycotoxins in tomatoes.); Plant extracts (certain plant extracts have been found to be effective in reducing the growth of patulin and *Alternaria* producing fungi. For instance, the use of essential oils such as clove and cinnamon oil has been found to be effective in reducing patulin levels in apples (Sivakumar and Bautista-Baños, 2014). Similarly, the use of garlic extract has been found to be effective in reducing *Alternaria* mycotoxins in tomatoes); and Enzymes (some enzymes such as polyphenol oxidase and peroxidase have been reported to reduce patulin levels in fruits (Chen et al., 2023). For example, polyphenol oxidase extracted from apples has been shown to effectively reduce patulin levels in apple juice. Some enzymes such as peroxidase and glucose oxidase have been reported to reduce *Alternaria* mycotoxins in fruits (El Hajj Assaf, 2018). For example, peroxidase extracted from horseradish has been shown to effectively reduce AOH levels in tomatoes (Tittlemier et al., 2020). Glucose oxidase produced by *Aspergillus niger* has been shown to reduce AOH levels in apples (Sun et al., 2023).

It is conspicuous to mention that the biological methods available to detect *Alternaria* and patulin in fruits, require specialized equipment and trained personnel, making them more costly and time-consuming than other methods such as visual inspection. False positives and false negatives can also occur if the sample is not properly prepared or the method is not sensitive enough to detect low levels of contamination. Additionally, the presence of other microorganisms or compounds in the fruit matrix may interfere with the detection of *Alternaria* and patulin (Bartholomew et al., 2021). In contrast, these biological methods are highly sensitive and specific, allowing for accurate detection and quantification of *Alternaria* and patulin in fruit samples. They are also able to detect low levels of contamination, which is important for ensuring the safety of fruit products for consumption (Barkai-Golan and Paster, 2008). These methods have been validated and widely used in food safety research and regulatory compliance programs.

7 Gaps and future prospects

There has been significant research on patulin and *Alternaria* toxins in fruit and derived products, there are still several gaps and future prospects for further research to better understand the occurrence, health effects, and control measures of these toxins. While there is a significant amount of research on the occurrence and levels of patulin and *Alternaria* toxins in certain fruits and derived products, such as apple juice and tomato products, there is a lack of information on the occurrence and levels of contamination in other fruits and derived products. For example, more research is needed on the occurrence and levels of patulin and *Alternaria* toxins in tropical fruits, such as mangoes and pineapples, and their derived products. There are inconsistencies in regulations and guidelines for patulin and *Alternaria* toxins in different countries and regions. For example, the European Union has set strict regulations for patulin in apple juice, while the United States has not set any specific regulations for patulin in apple juice. This can create confusion for consumers and manufacturers, and more harmonization of regulations and guidelines is needed. There is a significant amount of research on the health effects and toxicity of patulin, there is limited information on the health effects and toxicity of *Alternaria* toxins. More research is needed to better understand the health effects and toxicity of these toxins, particularly in humans. Analytical methods for the detection and quantification of patulin and *Alternaria* toxins have improved significantly in recent years, but there is still a need for more sensitive and specific methods. This is particularly important for detecting low levels of contamination in fruits and derived products. Moreover, There is a significant amount of research on control measures and mitigation strategies for patulin and *Alternaria* toxins in some fruits and derived products, such as apple juice and tomato products, there is a lack of information on control measures and mitigation strategies for other fruits and derived products. More research is needed to identify effective control measures and mitigation strategies for a wider range of fruits and derived products. There is also a need to investigate emerging mycotoxins in fruits and derived products, which may pose a risk to human health. Some examples of emerging mycotoxins include *beauvericin* and *enniatins*, which are produced by fungi of the genera *Fusarium* and *Aspergillus*.

8 Conclusions

It is clear that fungal infections and mycotoxins in fruits and derived products pose a significant risk to human and animal

health, making their detection and control crucial. While traditional methods for detecting and removing mycotoxins have been developed, there are still some challenges that need to be addressed, such as the simultaneous detection of multiple mycotoxins and the limitations of physical and chemical methods for mycotoxin removal. Biodegradation has emerged as a potentially viable alternative strategy for mycotoxin control due to its high efficiency, specificity, and lack of pollution. However, further research is needed to understand the complex mechanisms involved in mycotoxin detoxification and to isolate and identify high-purity enzymes for mycotoxin degradation. Advances in genetic engineering may also play a crucial role in this area in the future. Overall, the development of accurate and rapid detection technology and effective mycotoxin control strategies is necessary to ensure the safety of fruits and derived products in human diets.

Author contributions

SB: Conceptualization, Methodology, Software, Formal analysis, Investigation, Writing - original draft, Data curation, Project admin- tration. JN: Funding acquisition, Conceptualization, Writing - review & editing, Visualization, Project administration. YL: Investigation, Writing - review & editing. GX: Writing - review & editing. SF: Material search and Data collection: LH. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Muhammad Musa Khan,
Zhejiang University, China

REVIEWED BY
Mohammad Sarraf,
Shahid Chamran University of Ahvaz, Iran
Mohammad Nauman Khan,
Huazhong Agricultural University, China

*CORRESPONDENCE
Shujaul Mulk Khan
shujaqau@gmail.com
Linda Heejung Lho
heeelho@gmail.com
Heesup Han
heesup.han@gmail.com

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Detoxifying the heavy metals: a multipronged study of tolerance strategies against heavy metals toxicity in plants

Ujala Ejaz[1], Shujaul Mulk Khan[1,2]*, Noreen Khalid[3], Zeeshan Ahmad[1], Sadia Jehangir[1], Zarrin Fatima Rizvi[3], Linda Heejung Lho[4]*, Heesup Han[5]* and António Raposo[6]

[1]Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan, [2]Member Pakistan Academy of Sciences, Islamabad, Pakistan, [3]Department of Botany, Government College Women University, Sialkot, Pakistan, [4]College of Business, Division of Tourism and Hotel Management, Cheongju University, Cheongju-si, Chungcheongbuk-do, Republic of Korea, [5]College of Hospitality and Tourism Management, Sejong University, Seoul, Republic of Korea, [6]CBIOS (Research Center for Biosciences and Health Technologies), Universidade Lusófona de Humanidades e Tecnologias, Lisboa, Portugal

Heavy metal concentrations exceeding permissible limits threaten human life, plant life, and all other life forms. Different natural and anthropogenic activities emit toxic heavy metals in the soil, air, and water. Plants consume toxic heavy metals from their roots and foliar part inside the plant. Heavy metals may interfere with various aspects of the plants, such as biochemistry, biomolecules, and physiological processes, which usually translate into morphological and anatomical changes. They use various strategies to deal with the toxic effects of heavy metal contamination. Some of these strategies include restricting heavy metals to the cell wall, vascular sequestration, and synthesis of various biochemical compounds, such as phyto-chelators and organic acids, to bind the free moving heavy metal ions so that the toxic effects are minimized. This review focuses on several aspects of genetics, molecular, and cell signaling levels, which integrate to produce a coordinated response to heavy metal toxicity and interpret the exact strategies behind the tolerance of heavy metals stress. It is suggested that various aspects of some model plant species must be thoroughly studied to comprehend the approaches of heavy metal tolerance to put that knowledge into practical use.

KEYWORDS

heavy metal stress, plant defense mechanisms, genomics and transcriptomics, cell signaling pathways, plant structural and functional biology

1 Introduction

Anthropogenic and natural activities have resulted in the vast concentration of heavy metals in the atmosphere, which affects humans and other living organisms (Rathoure, 2020; Khalid et al., 2021; Yan et al., 2022). Rapid industrialization, intensive mining processes, and extensive agricultural activities plays crucial role in contaminating the

environment with heavy metals (Pant and Tripathi, 2014). Heavy metals polluted soils and water bodies have far ranging effects on various aspects of plant life (Parmar et al., 2013), which include but are not only limited to morphology (Chatterjee and Chatterjee, 2000; Smeets et al., 2013), anatomy (Liza et al., 2020; El-Shabasy, 2021), physiology (Asai et al.), and cell signaling (Calderini et al., 1998; Bartels and Sunkar, 2005). Several plants can cope with high levels of heavy metals, which are generally termed as tolerant species, for example, *Erigeron Canadensis, Arundo donax* L, *Populus alba and Morus alba* (Ahmad et al., 2019b) *Sporobolus diander, Cynodon dactylon, Brachiaria mutica, Digitaria ischaemum, Digitaria longiflora, Eragrostis cynosuroides, Launaea asplenifolia, Stylosanthes scabra* and *Parthenium hysterophorus* (Gautam and Agrawal, 2019) can grow, survive, and exhibit greater tolerance to heavy metals but some species show detrimental symptoms of heavy toxicity, like *Malvestrum coromandelianum, Alternanthera paronychioides, Cyperus rotundus, Ambrosia chamissonis, Hyptis suaveolens Xanthium strumarium* and *Portulacca olearcea* (Gautam and Agrawal, 2019). Many studies are available on the impacts of heavy metals toxicity on plants, and various strategies that were used to overcome the negative impacts of this toxicity such as using biochar can reduce the bioavailability of heavy metal ions in soil environments as well as retain, stabilize, and inactivate toxic heavy metals. (Wang et al., 2020; Mansoor et al., 2021; Yang et al., 2022d; Elkhlifi et al., 2023). Another such example of using chelating agents like EDDS, EDTA, organic and synthetic chelators in soil to reduce the toxic effect of heavy metals in plants (Chen et al., 2020; Gluhar et al., 2020; Yang et al., 2021; Ejaz et al., 2022; Gavrilescu, 2022). There are several review papers available on this subject, most of them focused on one or two aspects of the plants but this review provides deep insight on various aspects from structural and functional biology to genetics, molecular, and cell signaling levels, which integrate to produce a coordinated response to heavy metal stress. It is necessary to study various aspects of plant life under heavy metals stress to decode the exact mechanism of the tolerance in plants. This review provides suggestions for future research on the subject as well as the practical uses of the knowledge we have obtained thus far.

The word heavy metal is usually controversial, and various authors have tried to define it by referencing the density of the metals that are involved (Duffus, 2002). We will define heavy metals in this paper based on their density, which ranges between 5.0g/cm^3 and atomic weight above 23 units for convenience (Koller and Saleh, 2018). Some heavy metals are fundamental components of biological systems, but most are toxic in higher concentrations (Asati et al., 2016). Some heavy metals are required, within permissible limits only, for plant growth, and these types of elements are called essential heavy metals. These include Zinc (Zn), Iron (Fe), Manganese (Mn), Cupper (Cu), Nickel (Ni) (Asati et al., 2016; Anum et al., 2019). The threshold range of heavy metals in plants, below which they are beneficial and above which they become toxic, varies depending on the specific heavy metal and the plant species. Zn is an essential micronutrient for plants and is beneficial for growth and development at low concentrations. However, excessive uptake of Zn can lead to toxicity, which may manifest as reduced growth and yield, chlorosis, and reduced root and shoot biomass. The threshold range for Zn in plants is generally considered to be between 20-100 ppm (Alloway, 2012). Fe is also an essential micronutrient for plants and is involved in a wide range of physiological processes, including photosynthesis, respiration, and nitrogen fixation. The threshold range for Fe in plants is generally considered to be between 50-500 ppm (Barker and Pilbeam, 2015). Mn is an essential micronutrient required for photosynthesis, enzyme activity, and nitrogen metabolism. The recommended range for Mn in plant tissues is between 15-100 ppm (Nagajyoti et al., 2010). Cu is required in small amounts for plant growth and development, but excessive uptake can lead to toxicity. Symptoms of Cu toxicity in plants include reduced growth, leaf curling, chlorosis, and necrosis. The threshold range for Cu in plants is generally considered to be between 4-15 ppm (Awashthi, 1999; Nagajyoti et al., 2010). Ni is an essential micronutrient required for urease enzyme activity and seed germination. The recommended range for Ni in plant tissues is between 0.1-1 ppm (Allaway, 1968; Awashthi, 1999; Nagajyoti et al., 2010).

These essential heavy metals are required in some biological roles as they act as co-factor of enzymes, part of enzymes, while others are required for redox reactions in plants. Whereas some heavy metals have no known biological functions in plants; therefore, they are not required by the plants, known as non-essential heavy metals, Mercury (Hg), Cadmium (Cd) and Chromium (Cr) are some non-essential heavy metal examples (Wuana and Okieimen, 2011). The threshold levels for non-essential in plant tissue are typically lower compared to the essential heavy metals. The recommended maximum limit for mercury in edible plant tissue is 0.01 mg/kg for cadmium is 0.3-1.0 mg/kg and for chromium is 0.1-1.0 mg/kg, depending on the plant species (Organization, W. H, 2009). Heavy metal concentration limits in cereal crops according to the World Health Organization (FAO/WHO, 2017), European Union (EU) (Regulation, 2006), and United States Environmental Protection Agency (EPA, U. S, 2019) for various heavy metals can be seen in Table 1.

Both natural and anthropogenic activities expose heavy metals in the environment, but anthropogenic activities are the core cause of most of the heavy metal pollution in the atmosphere (Smiljanic et al., 2019). Weathering different types of rocks and minerals found in the Earth's crust results in various heavy metals but usually within acceptable limits (Chatterjee and Chatterjee, 2000). There are two types of soil in which heavy metal pollution is produced, including point and non-point sources. Point sources are pollution from discrete source, such as pipes or effluent outfalls. Non-point sources are sources with no discrete source, and the pollutants enter the environment *via* many pathways (Rehman et al., 2008). Both natural and anthropogenic sources may be point or non-point in nature. Natural elements from a parent substrate reach the soils during pedogenetic processes. Heavy metals in soil depend on the parent substrate's geology (Öncel et al., 2000). Fuel consumption with transportation, homes, and industries usually releases Zinc (Zn), Lead (Pb), Cadmium (Cd), and Chromium (Cr). Relatively high levels of Cadmium (Cd), Arsenic (As), Lead (Pb), and Nickel (Ni) are observed in the exhaust and non-exhaust releases from

TABLE 1 Recommended concentration limit of heavy metals in different crops and plant species.

Heavy metal	Crop/plant species	WHO maximum limit (mg/kg)	EU maximum limit (mg/kg)	EPA maximum limit (mg/kg)
Arsenic (As)	Rice	0.2	0.2	0.01
	Leafy vegetables (spinach, lettuce, etc.)	0.1	0.1	0.4
	Root vegetables (carrots, potatoes, etc.)	0.1	0.1	0.4
Cadmium (Cd)	Rice	0.2	0.05	0.4
	Leafy vegetables (spinach, lettuce, etc.)	0.1	0.1	0.3
	Root vegetables (carrots, potatoes, etc.)	0.1	0.1	0.3
Chromium (Cr)	Leafy vegetables (spinach, lettuce, etc.)	N/A	0.5	1.1
Copper (Cu)	All crops	N/A	50	15-250
Mercury (Hg)	Rice	0.02	0.02	0.1
	Leafy vegetables (spinach, lettuce, etc.)	0.02	0.02	0.1
	Root vegetables (carrots, potatoes, etc.)	0.02	0.02	0.1
Lead (Pb)	All crops	0.3	0.1	0.1
Zinc (Zn)	All crops	N/A	100	N/A
Iron (Fe)	Wheat	–	–	500
	Rice	–	–	500
	Barley	–	–	500
Manganese (Mn)	Wheat	100	–	–
	Rice	50	–	–
	Barley	50	–	–

Provided by: WHO; FAO, Food and Agriculture Organization; EC, European Union Standards.

vehicles (Lee et al., 2021). The burning of coal releases Arsenic (As), Cadmium (Cd), and Lead (Pb) (Ali et al., 2019). A number of industries release their effluents that contain heavy metals directly into various water bodies from where the heavy metals enter soil and food chains (Rehman et al., 2008). Zinc (Zn), Iron (Fe), Lead (Pb), and Manganese (Mn) are released due to the burning of hair and effluents of the tanning industries (Hashem et al., 2017). Heavy metals can accumulate in plants when they are grown in contaminated soils or exposed to polluted air and water. When humans consume these contaminated plants, they can be exposed to high levels of heavy metals, which can lead to serious health problems. Heavy metals such as lead, cadmium, arsenic, and mercury are particularly concerning due to their toxicity and ability to accumulate in the body. When humans consume plants containing these heavy metals, they can experience a range of adverse health effects. For example, lead can cause neurological damage and developmental delays in children, while cadmium can damage the kidneys and increase the risk of cancer. Arsenic exposure has been linked to skin, lung, and bladder cancer, and mercury can cause neurological damage and developmental delays in children (Mudgal et al., 2010; Gall et al., 2015).

Plants possess a significant ability to absorb and store pollutants in their tissues. The mechanisms of metal uptake and transfer to different parts of plants have been the subject of various researchers (Ma et al., 2010; Rastogi et al., 2017; Ul Haq et al., 2020; Ihtisham et al., 2021). Heavy metals are usually present in the state of ions or precipitates in the soil that plants facilitate to induce the pH change of the soil and the production of chelators (Adamczyk-Szabela et al., 2015; Yang et al., 2022b). Essential and non-essential heavy metals have similar structural characteristics, which makes it difficult for plants to distinguish between the two metal classes. Thus, the root hairs ingest essential and non-essential metals from soil sap, where their concentration is usually higher than the epidermal cell sap. The soil sap enters the epidermal cells using a symplast pathway, which crosses into cortical cells *via* the plasmodesmata. The sap enters from cortical cells through the apoplast pathway to the stele

through the plasmodesmata. The cell sap can move through the plasmodesmata and enter the root's xylem cells (Meers et al., 2009; Tangahu et al., 2011). The plasma membrane of epidermal cells has several channel proteins and pumps for the uptake. These include a) proton pumps, which are special ATPases that use energy to generate electrochemical gradients, b) co and anti-transporters that uptake metals using electrochemical gradients that are generated by proton pumps, and c) carrier proteins that transfer ions into the cells (Ardestani and Van Gestel, 2013).

Heavy metals move upwards from the roots to the other sections of the plant along the xylem stream. Atmospheric heavy metals are usually released as aerosols, and vapors are filtered by the leaves from the atmosphere (Shahid et al., 2017). There are morphological traits, such as cuticle thickness, the stomatal, and the surface area of leaf (Barber et al., 2004; Larue et al., 2014) and physicochemical traits of the heavy metals, such as the density, size of the ion, and solubility of the metal ion (Xiong et al., 2014). A report shows that vegetables that grow near heavy industries have a higher content of heavy metals in their leaves (Shahid et al., 2013). The uptake of heavy metals occurs through the stomatal pores, which are cracks in the cuticle, ectodesmata, which are special channels in between the auxiliary cells and guard cells of the epidermis, and aqueous pores (Fernández and Brown, 2013). Many studies show that the foliar absorption of heavy metals is dose dependent, and there is a linear relationship between many heavy metals concentration in the air and their concentration inside the leaves (Kozlov et al., 2000; Bondada et al., 2004; Fernández and Eichert, 2009).

2 Impact of heavy metal on plant's structural and functional biology

Plants undergo different morpho-physiological and anatomical changes during oxidative stress (Li et al., 2021; Li et al., 2022a; Noor et al., 2022; Sun et al., 2022). Heavy metals interact with plants in two ways. First, heavy metals compete with essential nutrients during root uptake from the soil, preventing plants from growing normally. Second, heavy metals enter the plant, disrupt its metabolism, and have toxic effects on its internal and external structure (Liza et al., 2020). Heavy metal concentrations above the permissible level will negatively affect plants directly and indirectly (Blaylock, 2000). The direct negative effects include inhibiting enzymatic activities *via* binding to the sulfhydryl group or a deficiency of certain metals in metalloproteins or metal protein complexes (Van and Clijsters, 1990). Another direct effect is the damage to cellular structures, such as chloroplast and mitochondria, due to oxidative stress (Jadia and Fulekar, 2009). High doses of certain heavy metals slow down the process of photosynthesis, transpiration, and the growth rate in various plants (Yadav, 2010). We will discuss the impact of heavy metal stress on plants' structure and functional biology in detail in this review.

2.1 Morphological changes in plants

Heavy metals in plants can visualize themselves *via* visible damage to the epidermal tissues of the roots, stems, and leaves. A study noted a reduction in the leaf thickness because of the increased size of the bulliform and endodermis cells, which forced a decline in the size of the parenchyma cells (Alfaraas et al., 2016). Another study recorded a reduction in the leaf lamina size, root, and shoot length of *Shorea robusta* due to Cd, As, and Pb contamination (Pant and Tripathi, 2014). A reverse effect was observed with Cd and Pb in some plants. The plant length of different parts increased, but the volume of these organs decreased (Zhang et al., 2020; Wu et al., 2021). Heavy metals are toxic to plants, which causes chlorosis, slows down plant growth, and a reduces yield (Singh and Kalamdhad, 2011). A previous study, which assessed the micromorphological changes in Taraxacum officinale due to heavy metal toxicity, observed a reduction in the leaf thickness and many more spaces among the cortex and other cells compared to the control group (Bini et al., 2012). An increase in the diameter of the root and shoot, the enlarged trichomes and salt glands, and a variation in the number of stomata are some of the morphological changes observed in *Catharanthus roses* grown in heavy metals contaminated soil (Soumya et al., 2022). Zn and Cd phytotoxicity in *Brassica juncea and Phaseolus vulgaris* causes a reduction in growth and development (Prasad et al., 1999). Morphological characteristics, fresh and dry weights, shoot/root length, leaf area, and leaf count of *Parthenium hysterophorus* were reduced due to the negative impact of Pb and Cd (Ejaz et al., 2022). Higher levels of Cr negatively impact the plant's total biomass, root and shoot growth (Alsafran et al., 2022). Dandelion (*Taraxacum officinale*) growing in contaminated soil, showed reduced leaf thickness and poor structural pattern of leaves and roots (Maleci et al., 2014).

2.2 Anatomical modifications in plants

The researchers studied dissimilarities in the internal structures of the plant's roots, stems, and leaves in response to heavy metals (Gomes et al., 2012; Noreen et al., 2019; El-Shabasy, 2021). A detailed overview of heavy metal's effects on the anatomy of various organs of plants is provided below.

2.2.1 Root and stem anatomy

Heavy metals penetrate the plants from the soil *via* the roots. The roots, therefore, receive more harmful effects than other parts of the plant. Parenchyma collapsed in a paddy plant's roots due to Pb and Cd-Pb treated plants (Alfaraas et al., 2016). Cd caused damage to the root's endodermis, epidermis, and cortex on the tissue level in non-resistive genotypes of rice plants, whereas no visible damage was observed in the resistant plants (Li et al., 2014). There was a 20-30% reduction in the root parenchyma and the size of parenchyma cells with a combined treatment of Cd-Cu ((Kasim, 2005). The visible negative consequences of Cd toxicity were noted on the lateral root, stem primordia and, general root architecture (Ronzan et al., 2018).

There are visible changes in the structure of various cell organelles and the pattern of cell division in reaction to heavy metals on the cellular levels. The researchers obtained the results for reducing cell division in apical root meristems of *Lamina minor* treated with Pb (Samardakiewicz and Woźny, 2005). Also, the chromosome morphology, such as anaphase bridges and chromosome stickiness, were affected similarly, which caused a reduced cell division in apical meristems of the roots in *Helianthus annuus* due to Cd toxicity (Jiang et al., 2000). A reduction in cell division due to Ni and Pb toxicity was observed for various other plants. The restricted cell division in the apical and lateral meristem of the roots is the interaction of these metals with chromosomes during the cell division (Kozhevnikova et al., 2007). They also observed the structural modification in root hairs, cell walls, and the vacuoles of the cells in the roots of cotton plants. Cell vacuoles generally increase in size, probably for the accretion of heavy metals that are absorbed by the roots (Daud et al., 2009). The researchers also observed structural modifications in the cell walls, vacuoles, and root hairs of the cell in the roots and stems of cotton plants. The thickening of the cell wall in *Vicia faba* was recorded in the roots and stem cells in heavy metal stress plants (Ronzan et al., 2018). The thickening of the cell wall is a resistant mechanism against heavy metal stress. The stems of some plants show xerophytic adaptations due to heavy metal toxicity, such as a thick cuticle over their epidermis, a thick cortex with a stone like an appearance, and general structural modification in the vascular bundles (Raju and Ramakrishna, 2021).

2.2.2 The anatomy of leaves

A leaf is the most fragile organ of the plant, which is severely damaged by environmental pollution (Dickison, 2000). Heavy metals can enter the leaf *via* the stomata or translocate from the roots *via* the stem. Heavy metals in the interior of the leaves have serious consequences for the leaf tissue as well as at the cellular level (Dickison, 2000; Yabanli et al., 2014). Due to the Cd treatment, the chloroplast was the most affected organ in Salix purpurea and Phragmites australis at the cellular level (Hakmaoui et al., 2007). Several grana and thylakoid membranes were negatively affected due to the leaf's heavy metal build-up. The cell showed a less developed vacuolar system at a high absorption of Cd (Malik et al., 1992; Ciscato et al., 1997; Hakmaoui et al., 2007). Heavy metal exposure in high concentrations also negatively affects cell division and the differentiation of newly developing leaves in addition to the chloroplast and vacuolar system (Cheng, 2003; Li et al., 2014). The leaf showed a thick cuticle with a wax deposition and expanded mesophylls (Raju and Ramakrishna, 2021).

2.3 Physiological changes in plants

Heavy metal stress has a great impact on the physiology of the plant. The researchers discovered that heavy metal accumulation in plants reduced biomass, chlorophyll, and photosynthesis activity, whereas proline and antioxidant enzymes increased. Various studies showed that the plant's soluble sugar content decreases as the concentration of heavy metal stress increase, particularly in crops. (Hemalatha et al., 1997) (Rascio and Navari-Izzo, 2011). Heavy metals influence many biological activities, including denaturing several enzymes (Ghori et al., 2019). The hyper activity of many enzymes, which include glucose-6-phosphate dehydrogenase and peroxidases, are linked to heavy metal toxicity in plant leaves (Van and Clijsters, 1987), which ultimately affects the stability of the cell membrane. The heavy metal accumulation of Ni, Cd, Cr, Ar, Pb, Ni, and others disturbs the plants' metabolic processes and physiological functions (Singh and Aggarwal, 2011). The proline amount in plant species increases under heavy metal stress, but the chlorophyll concentration decreases (Ahmad et al., 2021). Excess zinc inhibits the germination of cluster beans (*Cyamopsis tetragonoloba*) and its growth, sugar, amino acid, chlorophyll, and carotenoid content (Manivasagaperumal et al., 2011). Zn causes phytotoxicity in plants if it exceeds the required nutrient level (Vries et al., 2007). A high Zn level in soil restrains numerous plant metabolic activities, which results in fast growth (Choi et al., 1996). It has been demonstrated that cadmium toxicity in plants decreases the ATPase activity in the cell membranes of wheat and sunflowers (Fodor et al., 1995; Tang et al., 2020). Under Cd stress, plants experience a decrease in physiological activities such as stomatal opening, leaf moisture content, and transpiration, which causes osmotically stressed conditions due to which plants experience severe physiological disorders (Alsafran et al., 2022; Javad et al., 2022). Cd also causes symptoms, such as chlorosis, oxidative stress, and the darkening of the roots, which can all be fatal (Di Toppi and Gabbrielli, 1999), (Mohanpuria et al., 2007), (Mohanpuria et al., 2007). Cadmium above the threshold level may cause quick death and disturb the enzymes structure and function in plants and microorganisms (Prasad et al., 1999; Yang et al., 2022c). A change in the efficiency of the catalytic enzymes in *Phaseolus vulgaris* occurs due to a high concentration of Cd and Zn (Van et al., 1988) (Romero-Puertas et al., 2004). Lead is a harmful metal that causes necrosis, chlorosis, limited plant growth, and a low yield (Malar et al., 2014). A study on the rate of cell division in heavy metals shows that when the concentration of the heavy metals increased, the cell division exponent decreased, demonstrating a negative effect of the heavy metal on cell division (Duan and Wang, 1995). Nickel is very toxic at a high level and is currently being studied due to its significant deposition in sediments across the globe (Singh et al., 2017). Numerous physiological changes, which include chlorosis and necrosis, are caused by too much Ni in the soil in many plant species, which is most notable in rice (Zornoza et al., 1999) (Rahman et al., 2005) (Das et al., 1997). Ni at toxic levels adversely affects plants by interfering with a variety of physiological processes, including nutrient deficiency, growth parameters, enzyme activities, and photosynthesis. (Naz et al., 2022) Mercury at high concentrations is highly toxic to plant cells. Different studies on mercury (Hg) toxicity demonstrated that it could cause visible damage and physiological issues in plants. It attaches to water transfer proteins, which results in the stomata shutting and blocking the water passage in plants. (Zhang and Tyerman, 1999) (Zhou et al., 2007). According to a study, high mercury concentrations slow mitochondrial activity by triggering reactive oxygen species (Messer et al., 2005) (Sheetal et al., 2016). It was observed that an excess amount of cobalt decreased the chlorophyll content in plants by studying high concentrations of cobalt in

cauliflower leaves. Manganese phyto-toxicity causes necrotic and chlorosis on the leaves and stems. Another warning sign is a crinkled leaf, which develops on young leaves, stems, and petiole tissue. (Wu, 1994) (Wu, 1994) (Elamin and Wilcox, 1986). A high concentration of Mn in plants shortens the shoot and root length (Arya and Roy, 2011). Mn toxicity was examined in peas (*Pisum sativum*), and it was discovered that chlorophyll a and b concentrations, the relative growth rate, and the photosynthesis rate decreased with an increased Mn level (Doncheva et al., 2005). However, slower plant development and a decrease in the chlorophyll content were observed in tomatoes (*Lycopersicon esculentum*), which was recorded by (Shenker et al., 2004). Arsenic inhibits growth, and it results in the discoloration and the wilting of plants (Cox et al., 1996). Arsenic similarly inhibits the plant height and leaf area in *Oryza sativa* (Marin et al., 1993; Abedin et al., 2002; Pan et al., 2023). Plants that grow in polluted land produce more proline. which is a survival mechanism towards heavy metal stress, but the amount of carotenoids and chlorophyll decreased (Ahmad et al., 2023). Heavy metal inhibits plant growth by altering its physiological and biochemical processes. It is consequently obvious from several research findings that heavy metal contamination has a severe impact on the physiology of plants.

3 The role of plant genomics and transcriptomics under heavy metal stress

3.1 Genomics

Specific structural genes control plant tolerance, so it is necessary to recognize, validate, and characterize the genes linked to heavy metal stress. Plant stress genes are generally divided into two groups: early functional and delayed functional. The early functional genes become active rapidly but only briefly, whereas the delayed functional genes are slowly and consistently induced. The ATPase (HMA) gene family is linked to the accumulation of heavy metals, transportation, and effective resistance in plants. HMAs can be divided into two main subgroups: the Pb/Zn/Cd/Co P1B-ATPase and the Ag/Cu P1B-ATPase, based on their preference for specific metal substrates (Axelsen and Palmgren, 2001). Eight P_{1B}-ATPases were discovered in *A. thaliana* (Williams and Mills, 2005). P_{1B}-type ATPases was additionally discovered in *Triticum aestivum, Hordeum vulgare, Arabidopsis halleri, and Thlaspi caerulescens* (Deng et al., 2013). Different HMA gene expressions in different tissues protect *Populus trichocarpa* from the heavy metal stress of Ag, N, Cd, Cu, Zn, Pb, Mn, and Co (Li et al., 2015). HMA8 genes have been expressed in high levels in heavy metal stress conditions, in HMA1 and HMA4 leaves, and in the HMA5.1 roots. Heavy metal uptake and more expression of the genes have a direct correlation. For instance, HMA3 overexpression causes improved Cd accretion in plant parts (Morel et al., 2009). Over expression of HMA5 has similarly been found in *Oryza sativa* growing under high Cu contaminated soil (Deng et al., 2013). *Aeluropus littoralis* regulates the H^+-ATPase gene to control its potential for the remediation of Pb and Hg metals. (Jam et al., 2014). High concentrations of Cd resulted in overexpression of the gene family serine acetyltransferase (SAT) in *Arabidopsis thaliana* (Howarth et al., 2003).

3.2 Transcriptomics

Numerous studies have been conducted on transcriptomics to understand gene expression in heavy metals. Transcription factors (TFs) come from multigenic groups and control the expression of numerous genes, known as the main regulators (Hu et al., 2022). TFs attach to the distinct locations of cis-acting elements in gene promoters to control gene expression (Wray et al., 2003). Many TFs groups have been identified that control how plants react to heavy metal stress, which includes E2F-DP, E2F-DP, AREB/ABF, CCAATDR1, MYB, CCAAT-HAP3, DREB1/CBF, EMF1, MADS, AP2/EREBP, C2C2-Dof, CCAAT-HAP5, bHLH, C2H2, C3H, C2C2-YABBY, C2C2-Gata, ABI3VP1, ARF, C2C2-CO-like, ARID, CPP, CCAAT- HAP2, SBP, WRKY, bZIP, HSF, MYC, HB, AtSR, TUB, and NAC (Singh et al., 2002; Shameer et al., 2009; Noman et al., 2017). The basic leucine zipper (bZIP) in *Arabidopsis thaliana* and *Brassica juncea* transcription factors are activated in response to Cd stress (Ramos et al., 2007). TFs were additionally discovered in *Arabidopsis halleri* under Cd stress (Weber et al., 2006). *A. thaliana* exposed to Cd toxicity had two additional TFs, ERF1 and ERF5, induced by the AP2/ERF superfamily (Herbette et al., 2006). *Brassica napus* under Cd stress induced different transcripts, such as miR156, miR171, and miR396a (Zhou et al., 2008). Various levels of Cd stress may carry on the differential TF expression in these plants. miR166 was discovered to be downregulated in modified miRNA due to Cd stress, whereas miR171, miR529, miR319, and miR393 were observed to be highly expressed in *Medicago truncatula* (Zhou et al., 2008). miR529, miR319, MiR171, and miR393 were discovered to be upregulated under high Hg stress in *Medicago truncatula* (Zhou et al., 2008). Other researchers reported that miR398 is downregulated in plants under Hg and Cd (Kuo and Chiou, 2011; Min Yang and Chen, 2013). 18 different miRNAs were discovered in *Oryza sativa* under As stress due to differential expressions (Liu and Zhang, 2012). A scientist reported 69 miRNAs in *Brassica juncea* (Srivastava et al., 2012). The plant's growth was positively influenced by altering the expression of miR167, miR319, and miR854 *via* the artificial application of JA and IAA in another study (Gupta et al., 2014). Rice similarly showed a differential expression of seven miRNAs, which are encrypted genes for the transportation of nutrients, transcription factors, induce apoptosis, phytohormones equilibrium, and cell expansion under oxidative stress (Li et al., 2010).

4 Molecular level response mechanisms of plants

Heavy metal toxicity in plants results in the induction of cellular defense strategies, such as transportation and detoxification of heavy metals in the vacuole, which produces heavy metal

transporters, amino and other organic acids, antioxidants, and phytochelatins (Noor et al., 2022). Plants need many metal ions for various biomolecules in plants. Some heavy metals, which are required in small quantities, are required by enzymes as co-factors and other biomolecules. However, non-essential heavy metals negatively affect plants by restricting vital functional groups or displacing important metal ions in the biomolecules (Parmar et al., 2013).

4.1 Heavy metal transporters

Heavy metal transporters are believed to play an important role in plants, which implies that these types of transporters might be vital in the resistance to heavy metals, induced toxicity. These metal ion transporters include CPx-type ATPases, zinc-iron permeases (ZIP), and macrophage protein (Nramp) (Williams et al., 2000). These transporters are believed to be involved in obtaining heavy metals for vital cellular functions and regulating them (Parmar et al., 2013). Another family of proteins is Nramp-, which is involved in the uptake of Fe and Cu-, and it has been discovered to increase the Cd sensitivity if the related gene is overexpressed in certain heavy metal stress conditions (Thomine et al., 2000). Many ZIP family members have been identified so far, and at least 15 genes of the ZIP family members are in the genome of *Arabidopsis thaliana.* The ZIP family transporters help in Zn, Cd, and Co transportation. A study on zinc transporters in *Arabidopsis thaliana* suggests that protein assists the Zn sequestration (Van Der Zaal et al., 1999). Many intracellular transporters, which include HMA, ABC, CDF, NRAMP, and CaCA, participate in the compartmentalization of heavy metals. Chelated metals inside the vacuole depend on the activity of the two families of ABC transporters, known as multidrug resistance associated proteins (MRP) and pleiotropic drug resistance proteins (PDR). In addition, PC-Cd (phytochelatin-cadmium) complexes are transported by HMT1 transporters, found in the tonoplast, and tonoplasts contain CaCA and NRAMP transporters, which help in the shift of heavy metals from the cytosol to the vacuole. Many eukaryotes have CDF transporters. They have been observed to transport Cd, Ni, Fe, Mn, Co, and Zn metal cations from the cytoplasm to the vacuole, illustrated in Figure 1 (Krämer et al., 2007; Montanini et al., 2007; Peiter et al., 2007).

4.2 Amino and other organic acids

Certain other essential biomolecules have been discovered to help with the resistance against heavy metal toxicity in addition to heavy metal transporters. Some of these biomolecules comprise of amino acids, organic acids, phytochelatins, and metallothioneins (Grill et al., 1987; Kägi, 1991; Homer et al., 1997; Rauser, 1999). Several amino acids in combination with other organic acids in plants help in the chelation of heavy metals. The amino acids include Ile and Trp (Pb), Leu and Gly (Cd), succinic, oxalic, butyric, and citric acid (Hg), Ser (Hg), Glu and Trp (Ni), Gly, Leu, and Asn (Se), Ser, Leu, and Asp (Cr), organic acids, malic acid and malonic acid (Cd), citric and malonic acid (Se), malonic acid (Pb), Thr and Asp (Sn), malic and oxalic acid (Cr), malic and malonic acid (Ni), maleic, and malonic and malic acid (Sn) (Kocaman, 2022). The accumulation of asparagine against Zn toxicity was observed in the roots of *Deschampsia cespitosa*. The possible mechanism involves the Zn-Asparagine complex formation to detoxify Zn (Smirnoff and Stewart, 1987). A number of Brassicaceae members have shown high concentrations of free histidine in the xylem response to an Ni accumulation (Krämer et al., 1996; Kerkeb and Kramer, 2003). The proline concentration rose in response to the Ni toxicity in three plants, which included *Walsura monophylla, Phyllanthus palwanensis*, and *Dechampetalum geloniodes* (Homer et al., 1997). A major portion of Zn was observed to be bound by asparagine and

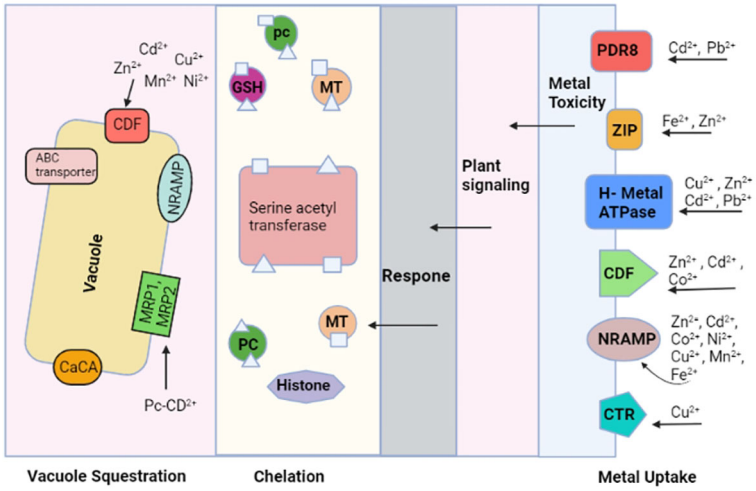

FIGURE 1
The biomolecules that are involved in uptake of heavy metals, chelation, and heavy metal sequestration/compartmentalization are shown. Many metal ion transporters are involved in this process. A high concentration of heavy metals in the cell initiates a defense response whereby heavy metal chelators are released, attached with heavy metals, and transferred in the vacuole.

proline in tomato and soya bean xylem sap (White et al., 1981). The plant releases particular amino acids in response to heavy metals, as shown in Table 2.

4.3 Phytochelatins

Plants and fungi release unique metal binding peptides called phytochelatins (PC) under heavy metal stress. PCs are an oligomeric form of glutathione with the attribute reappearance of the (-Glu-Cys)n-Gly [(PC)n], where n = 2–11 dipeptides of glutamate and cysteine (Ahmad et al., 2019a). Several heavy metals form PC complexes, but the most abundant ones are the PC complexes that involve Cd^{+2} and Cu^{+2}. Other relatively less abundant PC complexes involve Pb, Hg, and Zn (Thumann et al., 1991; Mehra et al., 1995; Maitani et al., 1996; Mehra et al., 1996). PCs are produced against heavy metals stress of Ag, Hg, Cu, Pb, Zn, and As. Heavy metals stress boost the PCs genes (LsPCS1) appearance in some plant species (He et al., 2005). The PCS1 gene in wheat was responsible for tolerance against Cd toxicity, and this gene could be used in the preparation of transgenic crops for heavy metal phytoremediation (Khan et al., 2020).

5 Heavy metal induced cell signaling in plants

Plants interact with heavy metals in two ways. First, plants are harmed by heavy metals. Second, they develop resistance mechanisms against them (Asati et al., 2016). Plants have many defense mechanisms against heavy metals. The sensing of heavy metal stress by plants initiates a number of responses on molecular and biochemical levels (Jalmi et al., 2018). Plants have three signaling pathways: the MAPK cell signaling pathway, calcium signaling, and hormone signaling in heavy metals (Jalmi et al., 2018).

5.1 MAPK pathway in heavy metal stress

A conserved evolutionary cell signal transduction module, called mitogen-activated protein kinase (MAPK), is involved in directing the extracellular cell signals to the nucleus to start suitable cellular responses. There are three components in the MAPK cascade, which include a) MAPK kinase (MAPKKK), b) MAPK kinase (MAPKK), and c) an MAPK. These components are connected through phosphorylation (Sinha et al., 2011). The MAPK signaling pathway is involved in mitosis especially during phragmoplast synthesis (Calderini et al., 1998; Yang et al., 2022a). The MAPK signaling transduction is extremely important to basic physiological functions, such as cell cycle regulation, abiotic stress signaling, and the defense mechanism (Tena et al., 2001). The accurate mechanism behind activation of this specific signaling pathway required a lot of investigation, but the heavy metal ligands and the reactive oxygen species (ROS) are the main factors that are responsible among the abiotic factors (Jonak et al., 2004; Smeets et al., 2013; Jalmi and Sinha, 2015). Heavy metals, such as Cd, Cu, and As induce MAPK signaling activation (Jonak et al., 2004; Yeh

TABLE 2 shows a number of amino acids that are released by plants against a particular heavy metal.

Plants	Heavy metals	Amino acids							
		Proline	Histidine	Asparagine	Aspartate	Threonine	Cysteine	Lysine	Ref.
Walsura monophylla	Ni	✓	–	–	–	–	–	–	(Homer et al., 1997)
Nepeta cataria	Pb	✓	–	✓	–	–	–	✓	(Zhou et al., 2020)
Solanum lycopersicum	Cu	–	–	–	✓	✓	–	–	(White et al., 1981)
Phyllanthus palwanensis	Ni	✓	–	–	–	–	–	–	(Homer et al., 1997)
Solanum lycopersicum	Cu	–	–	–	✓	✓	–	–	(White et al., 1981)
Alyssum lesbicum	Ni	✓	–	–	–	–	–	–	(Krämer et al., 1996)
Brassica juncea	Ni	–	✓	–	–	–	–	–	(Parmar et al., 2013)
Deschampsia cespitosa	Zn	–	–	✓	–	–	–	–	(Smirnoff and Stewart, 1987)
Phyllanthus palwanensis	Ni	✓	–	–	–	–	–	–	(Homer et al., 1997)
Solanum lycopersicum	Zn	–	✓	–	–	–	–	–	(White et al., 1981)
Arabidopsis thaliana	Cd	–	–	–	–	–	✓	–	(Domínguez-Solís et al., 2004)

et al., 2007; Ding et al., 2011; Rao et al., 2011; Smeets et al., 2013). However, very limited literature is still available about the response produced by other elements, such as Fe, Pb, and Zn. The exact mechanism against particular heavy metals is not yet understood in addition to this, but the researchers have investigated the pathways that are involved in a number of species. The MAPK cell signaling pathway is especially important in regards to mitigating heavy metal stress in number of plants. The exposure of *Medicago sativa* seedling, Cd, and Cu stress trigger four distinct MAPKs, which include a) SIMK, b) MMK2, c) MMK3, and d) SAMK. All four MAPKs increased their activities with an increase in the concentration of $CdCl_2$ and $CuCl_2$ (Jonak et al., 2004). MPK6 and MPK3 are the best known MAPKs in Arabidopsis that trigger stimuli, such as $CdCl_2$ and $CuSO_4$ (Asai et al, 2002; Pitzschke et al., 2009; Liu et al., 2010; Ahn et al., 2011; Beck et al., 2012). *Oryza sativa* increases the transcription of OsWJUMK1 (OsMPK20-4 homolog), OsMSRMK3 (OsMPK7 homolog), and OsMSRMK2 (OsMPK3 homolog) when treated with Cd and Cu (Rao et al., 2011; Beck et al., 2012). Other heavy metals also similarly induce MAPK cascades in Cu and Cd, but their mechanism is still not very widely investigated. For example, an Al ion sensitive yeast mutant showed an over expression of the MAPK gene, which suggests an alliance of the MAPK gene with an Al confrontation (Schott and Gardner, 1997). Al resistance is achieved in wheat roots with the induction of a 48kDa MAPK signaling transduction. This shows a link between Al stress and MAPK activation (Mossor-Pietraszewska, 2001). Myelin basic protein (MBP) was found to be activated in rice by a 42kDa MAPK, which is due to iron stress. Pre-treatment with glutathione (GHS) of the root apical cells in rice decreased the apical cell's death and reduced ROS-induced MAPK signaling (Tsai and Huang, 2006). The SIMPAK3 gene was significantly induced in tomatoes under Cd^{2+} stress. This strategy would increase the leaf's chlorophyll content and the root's biomass along with increased root activity, which all helped in Cd stress (Muhammad et al., 2019). Several biomolecules activate the MAPK pathway under heavy metal stress. These biomolecules include nitrogen oxide (NO), reactive oxygen species (ROR), and various plant hormones. such as auxins, ethylene, and abscisic acid (ABA) (Li et al., 2022b).

5.2 Calcium signaling under heavy metal stress

The Ca^{+2} signaling pathway is very complex in nature with various biomolecules that have varied roles in this pathway. Let's first look into the components of this signaling pathway and then the significance of this signaling pathway in heavy metal stress. The sensor proteins, such as calcineurin B-like protein (CBL)-CBL, calmodulin-like proteins (CMLs), calcium dependent protein kinases (CDPKs), calmodulins (CaMs), Ca^{2+}/CaM dependent protein kinases (CCaMKs), and interacting protein kinase (CIPK) modules identify the signatures of Ca. This results in physiological responses, such as metabolic pathways, ion transport, and gene regulation (Zeng et al., 2015; Kudla et al., 2018). The second phase consists of the responding molecules, such as the CIPKs and CDPKs. This type of signaling helps develop tolerance towards various stresses (Tripathi et al., 2009; Li et al., 2012; De La Torre et al., 2013). Several researchers reported ease in heavy metal stress in plants when Ca^{2+} was exogenously applied. Treatment with Cd has been shown to enhance the antioxidant enzyme activity, which includes the antioxidant enzyme activity of ascorbate peroxidise and glutathione reductase, which a reduction in the activities of these enzymes was achieved under exogenous application of Ca^{2+} (Ahmad et al., 2015). The application of Ca^{2+} to a sesame plant induced the upregulation of the acquired systemic tolerance system, such as antioxidant enzymes and lipid fractions to protect the membrane integrity (Makadia and Siegel, 2011; Abd_Allah et al., 2017; Chen and Wang, 2021). Some studies examined the effect of the exogenous Ca^{2+} application on the toxicity of heavy metals in plants, but the exact mechanism of the signal transduction through the calcium signaling pathway is still not very clear. Researchers have also identified potassium (K) as a regulator of calcium (Ca^{2+}) signaling pathways (Assaha et al., 2017; Johnson et al., 2022).

5.3 Hormone signaling under heavy metal stress

Phytohormones are tiny molecules, usually derived from secondary metabolities, used in biological processes like cell division, cell differentiation, cell elongation, growth and metabolism (Jaillais and Chory, 2010; Davies, 2012; Zluhan-Martínez et al., 2021). Several plant hormones are being produced in various plant organs under different conditions in varied concentrations. The most prolific of these hormones are auxin (IAA), cytokinins, abscisic acid, gibberellin, ethylene and brassinosteroid. A brief discussion of two of these hormonal signaling pathways and their importance in heavy metal stress is examined in the following passages.

5.3.1 Auxins

Auxin (Indole-3-acetic acid; IAA) is a vital hormone in plant growth and expansion. There are a number of important hormones within the auxin family, such as Indole-3-butyric acid (IBA), IAA, and NAA. Auxin helps plants in regards to creating a response to heavy metal toxicity by regulating its biosynthesis, degradation, signaling, and transport (Potters et al., 2007). Auxin plays significant functions in the root development both in normal and stress conditions. An important protein, PIN1, has been reported to influence the redistribution of auxin under Cu stress both in meristematic and elongation zones in the primary root of *Arabidopsis thialana* (Yuan et al., 2013). Several studies show that the endogenous synthesis of auxins is affected by heavy metals stress. These reports showed heavy metals stress correlations with auxin biosynthesis (Srivastava et al., 2013). It was observed that Cd induced nitrogen oxide (NO) concentration inhibits auxin transport under Cd stress, and it causes a reduction in the meristem size of the root. NO is also important in the auxin signaling pathway in Cd stress (Pető et al., 2011; Yuan and Huang, 2016). Heavy metal accumulation is generally a main

aspect in reducing endogenous auxin production. *Brassica juncea* in heavy metal stress, which decreases the endogenous production of three auxins, was noted (Srivastava et al., 2013). The IAA production was similarly disturbed due to the Cd stress in barley roots (Zelinová et al., 2015).

The exogenous application of auxin improved tolerance in aux1 As toxicity in transformed plants implies the vital function of auxin transportation and signaling method in heavy metal stress (Krishnamurthy and Rathinasabapathi, 2013). An increase in the roots and stem growth of a sunflower was observed under moderate Pb stress after the addition of IAA (Liphadzi et al., 2006). A comparative study was conducted on the effect of L-TRP, which is an ancestor of auxin, on the seedlings of *oryza sativa* grown in Cd polluted soil. The study noted that better growth and yield was obtained in the L-TRP treated seedlings compared to the control group (Farooq et al., 2015). A number of other researchers noted similar synergistic effects of exogenously applied auxins and their precursors on heavy metals tolerance in plants and their possible use in regards to enhancing the phytoremediation capacity of plants, but the exact mechanism behind the better tolerance, which is due to the exogenous application of the hormone, is still not clearly understood. The possible mechanism may involve an association between the miRNAs and auxins during heavy metals stress (Srivastava et al., 2013). It is necessary to mention that a complex interaction is concerned with the endogenous synthesis of auxins under heavy metals stress with a possible crosstalk between various signaling pathways. Further exploration of the subject will help in regards to understanding the underlining mechanism of the auxin signaling pathway and its role in heavy metals tolerance in various plants.

5.3.2 Abscisic acid

The abscisic acid (ABA) hormone plays an important role in different stages of plants, such as seed dormancy and ripeness (Nambara et al., 2010). ABA also helps tolerate many environmental stresses, such as drought (Leung and Giraudat, 1998). The ABA signaling pathway controls abiotic stress (Bartels and Sunkar, 2005; Danquah et al., 2014). The concentration of abscisic acid increases with an increase in abiotic stress, which indicates that the plant cells can settle in the harsh environmental conditions as necessary. ABA signal transduction comprises a core signaling pathway that has Snf1-related protein kinases 2 (SnRK2s), type 2C protein phosphatases (PP2Cs), and PYL ABA receptors (Ng et al., 2014). The researchers show that ABA concentration increases in response to heavy metal toxicity (Rauser and Dumbroff, 1981; Poschenrieder et al., 1989). High amounts of ABA were observed in *Typha latifolia* and *Phragmites australis* due to heavy metal exposure (Fediuc et al., 2005). Similar results were obtained by (Stroiński et al., 2010) for potato tuber and by (Kim et al., 2014) for rice. A solution of Hg, Cd, and Cu was applied separately during the growth of wheat seeds, and the ABA level increased with a high accumulation of heavy metals (Munzuroğlu et al., 2008). The cucumber seedlings observed a reduction in growth and an increased level of ABA under Cu and Zn stress (Wang et al., 2014). Contamination show the expression of ABA synthesis related genes in *Oryza sativa*, such as OsNCED3 and OsNCED2, which is the directive of four ABA signaling genes. A entire genome study of rice root bare to vanadium (V) showed a strong demonstration of ABA signaling related genes (Lin et al., 2013). The transcriptional control of ABA signals transduction during cucumber seed germination under Cu and Zn stress showed that in total nine PLY, two SnRK2, and three PP2C genes were involved in the ABA signal transduction (Wang et al., 2014). The above mentioned pathways can be seen in Figure 2.

5.4 Mechanism of tolerance against heavy metals toxicity

We conclude that plants use the following mechanisms for tolerance against heavy metals in light of the above discussion.

5.4.1 Role of plant's roots in heavy metal uptake

Plants have evolved several mechanisms to create barriers and reduce the uptake of heavy metals through their roots. One such mechanism is exclusion, where plants restrict the entry of heavy metals into the root system through the formation of Casparian strips, which are suberin-like layers that surround the endodermal cells of the root. This prevents the passage of heavy metals into the vascular tissue. Plants can also release organic acids and other compounds from their roots that can react with heavy metals in the soil, forming insoluble complexes that are less available for uptake by the plant (Fahr et al., 2013). Some plants can actively pump heavy metals out of their roots using ATP-dependent transporters, a process called active efflux. Additionally, plants can reduce the uptake of heavy metals by competing with other nutrients for absorption sites on the root surface.

5.4.2 Function of plant cell wall in metal tolerance

It has been reported that bivalent and trivalent metal cations bind to various functional groups, such as -OH, –SH, and –COOH to restrict the heavy metal to the cell wall, which is due to the presence of the carboxyl group in pectin of the cell walls (Mehes-Smith et al., 2013). Several heavy metals are known to accumulate in the cell wall of the epidermal cells of *humilis* and *Silene vulgaris ssp*, and the metals usually bind to pectin or silicates (Bringezu et al., 1999). The cell wall acts as a physical blockade to the entrance of heavy metals in the cell. However, it is interesting to note that it is still not clear how heavy metals are restricted in the cell wall.

5.4.3 Plasma membrane as a barrier towards heavy metals entrance to the protoplast

The plasma membrane contains a number of heavy metal transporters, which are very helpful with the tolerance against heavy metal toxicity. They both act as channels for the intake of essential and non-essential heavy metals as well as induce sensitivity against heavy metals toxicity (Solioz and Vulpe, 1996; Williams et al., 2000; Parmar et al., 2013). The metal transporters are of practical importance for phytoremediation, and they are important

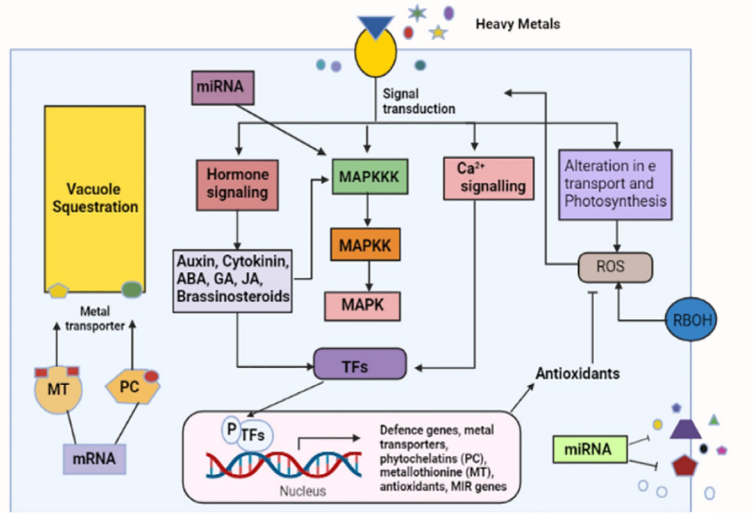

FIGURE 2
Crosstalk of the signaling pathways and the response created under heavy metals stress. The figure shows a number of signaling components working during heavy metal stress. Firstly, a high concentration of heavy metals is sensed, which initiates a cell signaling network that causes the activation of various metal responsive transcription factors.

for the tolerance against heavy metals toxicity (Ahmad et al., 2019b).

5.4.4 Phytochelation

The excretion of phytochelatins by plants against heavy metals is the best strategy that plants use. High affinity ligands, such as PCs, bind to metal cations to immobilize them and restrict the metals cations from interfering with the cells' biochemical pathways and cell signaling (Thumann et al., 1991; Maitani et al., 1996).

5.4.5 Compartmentalization/Detoxification of heavy metals

The plants either transport a heavy metal out of the cell or restrict it to the vacuole and then detoxification occurs once a heavy metal enters the cytosol, a process called sequestration/detoxification (Kanoun-Boulé et al., 2009; Singh et al., 2011). Thus, heavy metals are restricted from interference with vital metabolic pathways. This process allows plants to survive under metal-contaminated areas without toxic effect. Several transporter families are involved in this process, which includes ABC, CDF, HMA, and NRAMP transporters. Several studies show high concentrations of Cd and Zn in the vacuole of a cell. For instance, the nickel hyperaccumulator *Alyssum serpytllifolium* gathered up to 72% of Ni in the vacuole (Brooks et al., 1980; Ernst et al., 1992).

6 Conclusion

Heavy metals contamination is one of the greatest threats to human health and the survival of other living organisms, including plants. This threat increases with increasing industrialization in developed and developing countries. Heavy metals are non-biodegradable, which remain in the environment. Decontaminating soils and water bodies from heavy metal contamination is an economically expansive process. Thus, recent research is focused on finding plants for the phytoremediation of heavy metals. However, most of the plants are prone to the negative effects of heavy metals toxicity, which affect their growth and yield and have far-ranging impacts on various aspects of these plants. Some plants employ certain strategies to cope with heavy metal toxicity. These strategies may include anatomical changes within the plant organs, such as thickening cell walls to inhibit heavy metals into the cells or physiological adaptations, such as sequestration or molecular responses. such as chelation. All these responses depend on cell signaling within the plants. The cell signaling pathways adjust according to the concentration and the type of heavy metal pollution.

7 Future prospects

It is recommended that the researchers should study some model plants, including their plant morphology, anatomy, physiology, molecular biology, cell signaling, and genetics under heavy metal stress. It will further answer how heavy metals trigger certain signaling pathways and how those signals are translated into morphological, anatomical, physiological, and biochemical responses. It is also necessary to recognize the genes that are accountable for controlling all of these processes. The factors that restrict or facilitate the uptake, translocation, and sequestration of heavy metal ions in plants and that have been genetically modified to have high biomass and rapid growth rate should be the focus of future research. This will enable the practical application of knowledge in forming transgenic types, which are more effective at phytoremediation and have better capacities for tolerance against the toxic heavy metals. We now have a better understanding of stress tolerant mechanisms with the development of novel omics technologies for cellular complexity research. Numerous stress-

related genes have already been discovered with this advanced sequencing technologies. Unexpected outcomes have been attained by genetically modifying metabolites, proteins, and heavy metal stress responsive genes. The full potential of phenomics and functional genomics must be utilized.

It may be possible to lessen the load of heavy metals on agriculture by using nano-particles for the adsorption and co-adsorption of heavy metal ions from irrigation water. Another crucial area that could aid in achieving environmental sustainability is using bio-indicator plants to monitor heavy metal hot spots. Further research investment is required to understand better the interactions between plants and microbes under heavy metal stress, as this information may help develop practical strategies for recovering soils polluted with heavy metals.

8 Limitations

This review focuses on various genetic, molecular, and cell signaling levels that work together to produce a coordinated response to heavy metal toxicity and deduce the mechanisms behind the tolerance. However, many important questions still need to be clarified because not all heavy metals cause the same physiological and biochemical reactions in plants. Similarly, how different plants react differently to various heavy metals. Because of these, it is challenging to determine a single stress-induced pathway that protects plants from all heavy metals. Good basic knowledge of the antioxidative mechanisms in plants is needed for much of the in-depth study.

Author contributions

UE: Conceptualization; Formal analysis; Investigation; Literature review; Validation; Visualization; Roles/Writing - original draft; SK and AR: Conceptualization; Formal analysis; Investigation; Project administration; Resources; Supervision; Validation; Visualization; Writing - review and editing; NK: Visualization; Writing - review and editing; Validation; ZA and SJ: Formal analysis; Investigation; Methodology; Validation; Visualization; ZF: Writing - review and editing; LL and HH: Writing - review and editing; Funding acquisition. All authors contributed to the article and approved the submitted version.

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In Memoriam

We dedicate this article to our teacher and an incredible Plant Scientist Prof Dr Habib Ahmad (TI), who left this mortal world on the night of 6-7th April 2021 due to COVID 19; while this paper has been accepted on 6-7th April 2023 on the occasion of his death anniversary.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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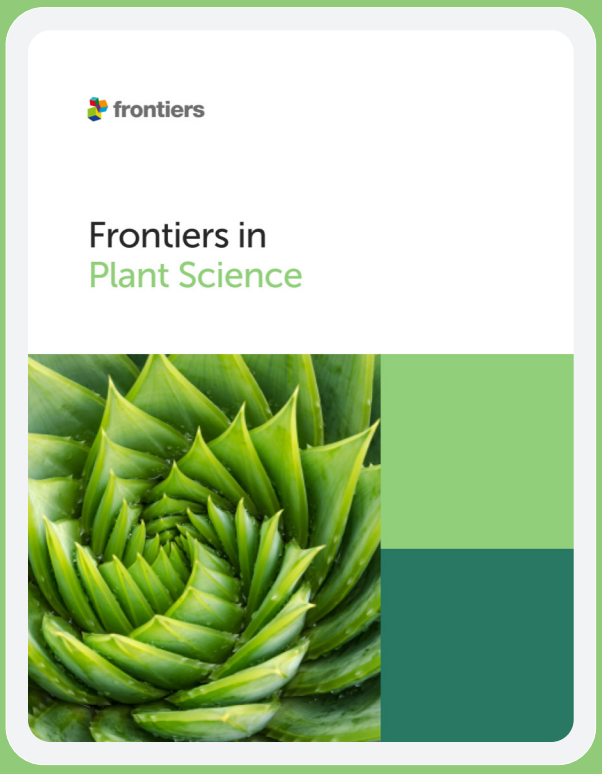

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