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# Physiological and metabolic responses of *Lolium perenne* L. roots to acid stress in cadmium-contaminated soil

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**Abstract:** Perennial ryegrass (*Lolium perenne* L.) has potential in the phytoremediation of cadmium (Cd)-contaminated soil due to its strong Cd accumulation capacity and high biomass. In this study, we investigated the growth physiology, Cd accumulation, and metabolites of *L. perenne* roots under different soil acid stress levels (pH 4.0, 4.5, 5.0 and 6.0) and Cd concentrations (100 and 0 mg/kg) after 90 days of growth. The results showed that soil acid stress significantly impacts the remediation capability and physiological metabolic properties of *L. perenne*. Based on root Cd content and enrichment coefficient, soil pH between 4.5 and 5.0 was more conducive to Cd accumulation. The growth physiology and Cd accumulation of *L. perenne* were inhibited under high soil acid stress (pH 4.0). High soil acid stress caused a decrease in root length, root volume, and root biomass of *L. perenne*. Root malondialdehyde (MDA) content and the activity of antioxidant enzymes (catalase (CAT) and peroxidase (POD)) increased significantly in response to high soil acid stress to enhance tolerance. Metabolomics analysis revealed that acid stress resulted in significant changes in certain metabolites. Tartaric acid, fructose and amino acids (glutamate and lysine) in the roots of *L. perenne* were compatible solutes under acid stress. This study indicated that *L. perenne* has strong physiological and metabolic tolerance, as well as Cd accumulation ability, in response to soil acid stress.

**Keywords:** adverse stress; heavy metal; metabolite profiling; soil pollution

Soil cadmium (Cd) pollution is one of the most important environmental problems in China and worldwide (Tan et al. 2021). Cd is one of the most toxic and transferable heavy metals. Most plants readily absorb Cd, which endangers human health through the build-up of the food chain (Ramana et al. 2021). Soil acidification affects Cd's chemical properties and activities, exacerbates Cd's toxicity to plants, and adversely affects plant growth (Xiang et al. 2018). Soil Cd pollution (Chen et al. 2024) and soil acidification (Duan et al. 2016) are particularly serious in southern China. Thus, it is imperative to find a solution to the issue of soil Cd pollution in acidic environments. Phytoremediation provides advantages, such as high efficiency, economically viable, and eco-friendly (Tan et al. 2023). For these reasons, it has attracted significant attention in the field of heavy metal pollution research (Yan et al. 2020, Liu et al. 2022, Oladoye et al. 2022). Hyperaccumulator plants enhance the removal capacity of heavy metals. More than 700 species

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of hyperaccumulator plants have been found worldwide, and several Cd hyperaccumulator plants have been screened from Cd-contaminated soil (Reeves et al. 2018). However, these plants generally lack the ideal qualities for soil heavy metal remediation: fast growth rate, high biomass production, and resilient environmental adaptability (Zhang et al. 2019b). There is little research on plants that can grow well on acidic soil in the south and efficiently remediate Cd-contaminated soil. Therefore, it is important to investigate plants that can remove cadmium pollution from areas with acidic soil.

*L. perenne* is a perennial ryegrass that belongs to the Gramineae. The plant shows a high potential for tolerance and accumulation of Cd (Zhang et al. 2019b, Jiang et al. 2022). It is widely distributed, easily cultivated, grows rapidly, and produces abundant biomass and root tissue. Therefore, *L. perenne* has important research value and broad application with respect to phytoremediation of Cd-contaminated soil (Zhang et al. 2019a). In recent years, with the increased severity of soil Cd pollution, metabolomics research on plant Cd stress response has become a new focus (Xie et al. 2019). The role of metabolomics in enhancing the potential of phytoremediation has been widely recognised by researchers (Oladoye et al. 2022). Tan et al. (2021) found that *Brassica juncea* roots had significant differences in amino acids, organic acid, carbohydrate, lipid, flavonoid, alkaloid and indole levels under Cd stress. Navarro-Reig et al. (2017) used non-targeted liquid chromatography-mass spectrometry (LC-MS) to study the effects of Cd stress on *Oryza sativa* L., and identified 97 metabolites with significant differences. This indicated that Cd stress severely affects the metabolism of amino acids, glycerides and carbon in *Oryza sativa* L. While the effects of soil acid stress on Cd accumulation and metabolic profiles in the roots of *L. perenne* are unclear. This study aimed to investigate the effects of soil acid stress levels and Cd concentrations on root growth, physiological resistance, Cd accumulation and metabolites of *L. perenne* in Cd-contaminated soil. It would provide a theoretical basis for the research of Cd accumulation in plants under soil acidification in southern China.

## MATERIAL AND METHODS

**Experimental materials and design.** The plant tested was *L. perenne*, a widely distributed plant that grows well in the acidic red soil area of south China. The seeds of *L. perenne* were cv. Yatsyn and purchased from Yuxi flower and bird market. The growth medium was well-mixed soil collected from the surface 0–20 cm profile in the Yuxi area (24°08'30"–24°32'18"N, 102°17'32"–102°41'37"E). The soil was air-dried, ground and sieved (5 mm) for the pot experiment. The soil type and soil texture were, respectively, red earth and clay loam. The soil pH, cation exchange capacity, organic carbon content, total nitrogen, total phosphorus and Cd background values were 4.5, 9.2 mmol/100 g, 0.55%, 1.09 g/kg, 0.40 g/kg and 0.14 mg/kg, respectively. The concentrated $H_2SO_4$, NaOH and $CdCl_2$ were obtained from Tianjin Chemical Reagents (Tianjin, China) to amend the soil pH and impose the treatments. Guaiacol, phosphate buffer (PB), $H_2O_2$ (60%, *v*/*v*), trichloroacetic acid, L-methionine, nitro-blue tetrazolium, ethylenediaminetetraacetic acid disodium salt (EDTA-2Na), riboflavin, thiobarbituric acid (TBA), $KMnO_4$ and oxalic acid were purchased from Macklin Reagents (Shanghai, China). These reagents were used to assess root activity and other metabolic processes.

The amount of $H_2SO_4$ and NaOH used was determined by pre-experimenting with soil acid and alkali treatment. According to the pre-experiment results, a certain amount of concentrated $H_2SO_4$ and NaOH per kilogram of soil was to adjust the soil pH to 4.0, 5.0 and 6.0. $H_2SO_4$ and NaOH were added to the tested soil (pH 4.5) in the form of a solution. We placed 4 kg of prepared soil in the pots (20 cm bottom diameter, 32 cm top diameter, 22 cm height). Diluted 4 mL of concentrated $H_2SO_4$ to 500 mL with distilled water, then added to the soil and stirred well. 1.042 g and 4.166 g NaOH were respectively dissolved in distilled water to a constant volume of 500 mL and then added to the soil and stirred evenly. After mixing, the soil was left standing for 30 days to diffuse and dissolve the acid and alkali fully and kept the soil moist (the soil water content at 80%) during the soil standing. The treated soil samples were air-dried to determine soil pH again with a pH meter. Four acid stress levels were set with soil pH of 4.0, 4.5, 5.0 and 6.0. The concentration of Cd was set at 100 mg/kg and 0 mg/kg. The Cd concentrations were selected in this study were based on previous relevant studies (He et al. 2018, Ramana et al. 2021). Also, we intended to amplify treatment effects and produce a robust response capable of detecting statistical differences. The method was to add a certain amount of $CdCl_2$ solution into the soil and mixed

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well to obtain our tested Cd treatments (Ramana et al. 2021). The pot experiment commenced after the prepared soil stood for 30 days to make sure the adjusted soil was very stable (Table 1). All pots were irrigated with distilled water (no Cd was detected in the water, pH 7.0) to keep the soil moist (Yi and Wang 2017, Ramana et al. 2021). There were eight treatments in total (four soil pH levels and two Cd rates), with three replicates per treatment. After the soaking treatment, 40 seeds were evenly sown in each pot of soil. Plants were cultivated in the greenhouse. The seedlings were thinned to 20 uniform specimens per pot after 15 days. During the growth of *L. perenne*, the soil water content was maintained at 80%, and no Cd was detected in the water. *L. perenne* grew under natural light, with a day-night temperature of 25/20 °C and a relative humidity of 66%. Hoagland's nutrient solution was added once every 7 days, at 200 mL per basin. Meanwhile, the prevention of diseases and insect pests was ensured, and weeds were removed from the pots. The plant was harvested after 90 days of growth (mature stage), and the relevant indices were determined by sampling.

**Measurements of growth parameters and Cd content in plant roots.** Intact roots of *L. perenne* were removed from the soil, washed and drained with distilled water, and used to measure root length and volume. Root activity, relative conductivity and malondialdehyde (MDA) content were determined using the triphenyltetrazolium chloride (TTC) method, conductometer method and thiobarbituric acid method, respectively (Yang et al. 2021, Zhao et al. 2021). The activities of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) of *L. perenne* roots, were determined using the nitro-blue tetrazole, UV spectrophotometry and guaiacol methods, respectively (Javed et al. 2017, Wang et al. 2020). Subsamples of root tissue were placed in an oven, dried at 105 °C for 15 min, and subsequently dried at 65 °C until constant weight. Root biomass was calculated by weighing the samples to the nearest ten-thousandth gram with an electronic balance. The dried root samples of *L. perenne* were ground and passed through a nylon sieve (0.25 mm), then a 0.5000 g subsample was digested in 10 mL of concentrated acid solution (9 $HNO_3$:4 $HClO_4$) (Ramana et al. 2021).

The air-dried soil samples were ground and passed through a 0.15 mm sieve and digested in $HNO_3$-HCl-$H_2O_2$. The Cd content of the digested solution was determined by atomic absorption spectrophotometry. The Cd enrichment coefficient of roots = root Cd content/soil Cd content. The Cd transfer coefficient = aboveground Cd content/root Cd content.

**LC-MS analysis.** Root extracts were prepared using the following procedures: fresh roots (100 mg) frozen in liquid nitrogen were homogenised into a fine powder using a mortar and pestle. The extraction solution was added (99.875% methanol acidified with 0.125% formic acid) and vortexed for 10 s while kept in an ice bath. Each sample was sonicated for 15 min at a maximum frequency (40 kHz) in a water bath at room temperature (20 °C). Each sample was then centrifuged for 10 min at maximum speed (20 000 × *g* for Eppendorf tubes; 3 000 × *g* for glass tubes). The supernatant was then stored at –80 °C until further LC-MS analysis. A 1290 UHPLC linked to a 6545 QTOF/MS system (Agilent Technologies, Santa Clara, USA) was used for the qualitative and quantitative analysis of significant metabolites in roots. HPLC grade acetonitrile, methanol and formic acid (Merck KGaA Co., Ltd, Darmstadt, Germany) were used for LC-MS analysis. Column chromatography (CC) used materials including SB-C18 (2.1 mm × 50 mm, 1.8 μm; Agilent Technologies) and BEH HILIC

Table 1. The pH and cadmium (Cd) content of experimental soil

| Cd treatment | Soil pH after 30 days of mixing | Soil pH after *Lolium perenne* was harvested | The soil Cd content after Cd treatment (mg/kg) |
|---|---|---|---|
| $Cd_0$ (0 mg Cd/kg) | 4.00 | 3.98 | 0.16 |
| | 4.50 | 4.50 | 0.14 |
| | 5.00 | 4.97 | 0.15 |
| | 6.00 | 6.03 | 0.18 |
| $Cd_{100}$ (100 mg Cd/kg) | 4.00 | 3.98 | 99.86 |
| | 4.50 | 4.48 | 99.73 |
| | 5.00 | 4.97 | 99.65 |
| | 6.00 | 5.96 | 99.17 |

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(2.1 mm × 100 mm, 1.7 μm, Waters). The mobile phase consisted of water containing 0.1% (*v*/*v*) formic acid (A) and methanol (B). The gradient elution was 0–14 min, 50–70% B; 14–64 min, 70–90% B; 64–79 min, 90% B; 79–84 min, 100% B; 84–94 min, 100% B. The scan range of MS/MS was positive ion mode and negative ion mode. The MS/MS fragments of the typical substances were acquired by collision energies optimised from 10, 20, and 40 eV, as described in a previous study (Wang et al. 2021). Quality control samples were prepared from an aliquot of 18 pooled samples. Compounds with accurate mass charge ratios (MS/MS) were annotated using METLIN (http://metlin.scripps.edu/) and HMDB (http://www.hmdb.ca/) databases. The metabolites were searched and annotated using "ID Browser Identification" in MPP software (Santa Clara, USA) with Metin PCDL-database (Agilent, Santa Clara, USA).

**Data analyses.** The effects of soil acid stress, Cd concentration and their interaction on root growth and physiological indices of *L. perenne* were examined using two-way ANOVA. Duncan's multiple comparison test was used to test the significance of differences among treatments. The data were analysed using R version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria). We used LC-MS data analysis, principal component analysis (PCA) and (orthogonal) partial least-squares-discriminant analysis (PLS-DA) to distinguish differences in metabolic profiles among experimental groups. Generally, these parameters, such as $R^2X$, $R^2Y$, and $Q^2$, were used to assess the quality and reliability of the established models. The model is considered to have excellent fitness and predictive capability when three parameters ($R^2X$, $R^2Y$, and $Q^2$) approach 1.0 (Tan et al. 2021). Differential metabolites between groups were selected using multidimensional coupling with single-dimensional analysis. In PLS-DA, the variable important in projection (VIP) was used to identify metabolites with biological significance. The significant metabolites were further verified using the Student's *t*-test. Root metabolites with VIP > 1.0 and $P < 0.05$ were selected as differential metabolites.

## RESULTS

**Root growth of *L. perenne*.** Table 2 shows that soil acid stress affected the root length, volume, and biomass of *L. perenne* ($P < 0.01$). In addition, Cd and its interaction with acid stress also significantly affected the root volume and biomass of *L. perenne* ($P < 0.01$). Across all pH treatments, root length, volume and biomass in Cd-polluted soil outgrew soil with no Cd (Figure 1). The only exception was root length within pH 4.0, which did not differ. Root length and volume in $Cd_0$ generally had the greatest values at pH 5.0 or greater. Although root length in pH 4.5 did not differ from pH 5.0 and 6.0. Root biomass across all pH treatments did not differ. In $Cd_{100}$ soil, the root length, root volume and root biomass of *L. perenne* at pH 4.0 were significantly lower than those of the other three acid treatments ($P < 0.05$) (Figure 1), indicating that high soil acid stress inhibits the growth of *L. perenne.*

**Root physiological resistance of *L. perenne*.** Soil acid stress and its interaction with Cd concentration had significant effects on root activity, root relative conductivity, root MDA, CAT and POD contents of *L. perenne* ($P < 0.05$) (Table 2). The root activity of

Table 2. Effects of acid and cadmium (Cd) on the root growth and physiological characteristics of *Lolium perenne*

| Index | pH | | Cd concentration | | pH × Cd concentration | |
|---|---|---|---|---|---|---|
| | *F* | *P* | *F* | *P* | *F* | *P* |
| Root length | 23.24 | 0.00** | 39.29 | 0.00** | 2.90 | 0.07 |
| Root volume | 31.46 | 0.00** | 312.02 | 0.00** | 7.47 | 0.00** |
| Root biomass | 34.00 | 0.00** | 326.55 | 0.00** | 18.13 | 0.00** |
| Root activity | 219.40 | 0.00** | 210.10 | 0.00** | 13.20 | 0.00** |
| Root relative conductivity | 17.20 | 0.00** | 107.50 | 0.00** | 3.99 | 0.03* |
| Root MDA content | 230.55 | 0.00** | 950.50 | 0.00** | 14.95 | 0.00** |
| Root SOD activity | 2.88 | 0.07 | 16.95 | 0.00** | 1.76 | 0.19 |
| Root CAT activity | 574.16 | 0.00** | 593.26 | 0.00** | 14.63 | 0.00** |
| Root POD activity | 98.31 | 0.00** | 2.31 | 0.15 | 3.84 | 0.03* |

*$P \leq 0.05$; **$P \leq 0.01$; MDA – malondialdehyde; SOD – superoxide dismutase; CAT – catalase; POD – peroxidase

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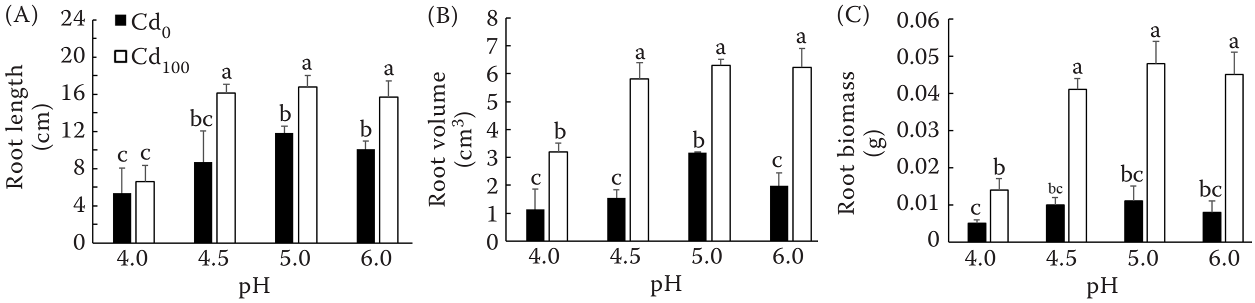


Figure 1. Effects of acid on the root growth of *Lolium perenne*. Data are the means ± standard error, $n$ = 3. Different letters indicate significant differences among treatments ($P$ < 0.05). $Cd_0$ – 0 mg Cd/kg; $Cd_{100}$ – 100 mg Cd/kg

*L. perenne* increased significantly with greater soil pH ($P$ < 0.05). In $Cd_0$ and $Cd_{100}$ soils, the root activity at pH 6.0 was nearly 2 and 7 times higher than at pH 4.0. This indicates that root activity of *L. perenne* was significantly inhibited under high soil acid stress (Figure 2A). Root relative conductivity was generally the lowest at pH 5.0, although the changes were more marked for $Cd_0$ than $Cd_{100}$ (Figure 2B). Root MDA content, CAT and POD activities decreased significantly as the soil pH increased ($P$ < 0.05) (Figure 2C, E, and F). Soil acid stress and its interaction with Cd concentration had no significant effects on root SOD activity ($P$ > 0.05) (Table 2, Figure 2D).

**Root Cd accumulation of *L. perenne*.** The effect of soil acid stress on Cd accumulation capacity in *L. perenne* is shown in Figure 3. Cd content in roots ranged from 370 to 828 mg/kg, while the Cd enrichment coefficient was 3.8 to 9.6 in the roots of *L. perenne* under different soil pH in $Cd_{100}$ soil. Cd content and enrichment coefficient more than doubled between pH 4.0 and 4.5. However, each variable showed a subsequent decrease at the two highest pH levels. The Cd transfer coefficient ranged from 0.19 to 0.32 and decreased significantly with increasing soil pH ($P$ < 0.05).

The results of the correlation analysis revealed that Cd content in the roots of *L. perenne* was significantly positively correlated with root length, root volume and root biomass and significantly negatively correlated with root CAT activity (Table 3).

**Root metabolites of *L. perenne*.** To understand the response mechanism of *L. perenne* roots to acidity, the acid stress (pH 4.0) group *vs*. control (pH 6.0) group was identified using Mass Profiler Professional (MPP) version B.15.01 software (Agilent Technologies, Santa Clara, USA). The identities

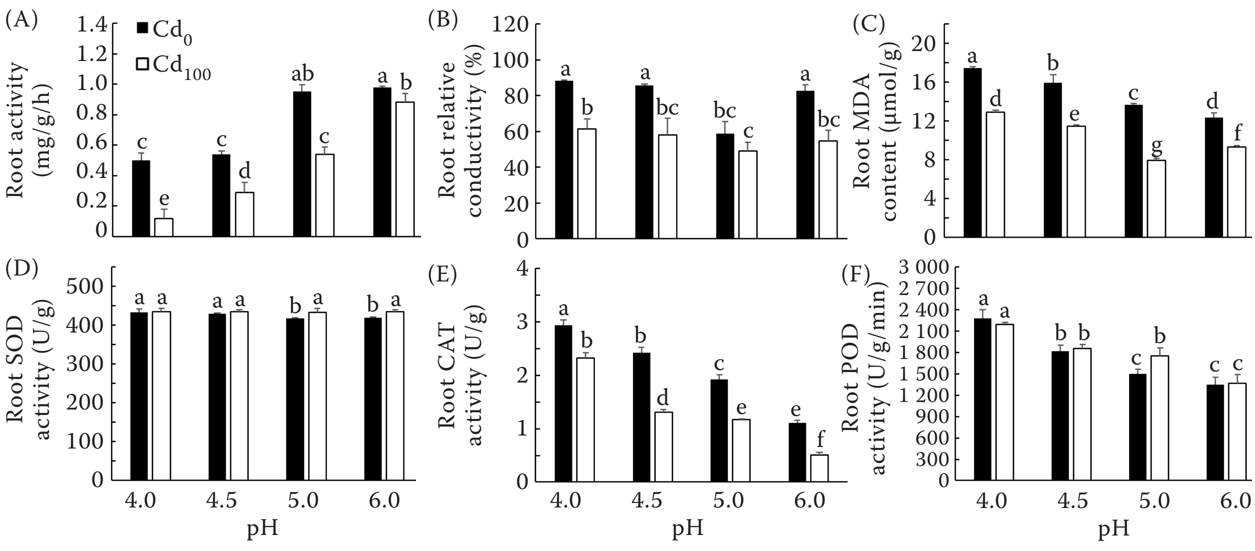


Figure 2. Effects of acid on the root physiological resistance of *Lolium perenne*. Data are the means ± standard error, $n$ = 3. Different letters indicate significant differences among treatments ($P$ < 0.05). MDA – malondialdehyde; SOD – superoxide dismutase; CAT – catalase; POD – peroxidase; $Cd_0$ – 0 mg Cd/kg; $Cd_{100}$ – 100 mg Cd/kg

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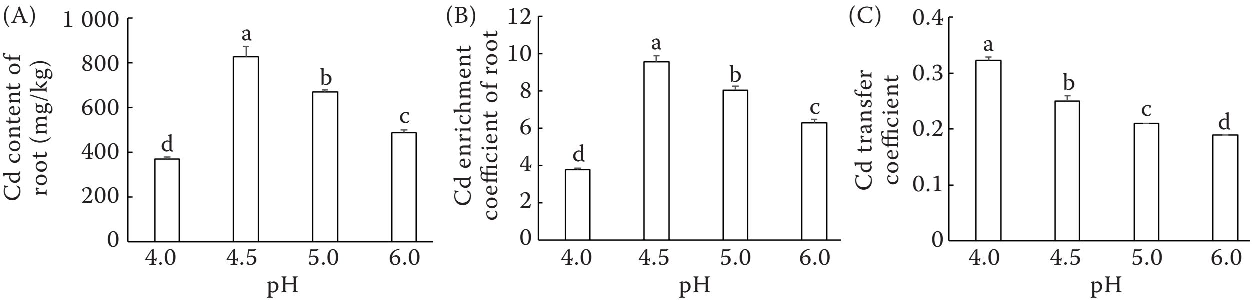


Figure 3. Effects of acid on the root cadmium (Cd) accumulation of *Lolium perenne* in $Cd_{100}$ (100 mg Cd/kg) soil. Data are the means ± standard error, $n$ = 3. Different letters indicate significant differences among treatments ($P$ < 0.05)

of metabolites were searched and annotated using "ID Browser Identification" in MPP software with Metin PCDL-database (Agilent). The metabolic changes in the roots of *L. perenne* were analysed using PCA and PLS-DA in both positive and negative ion modes. The PCA results (Figure 4A, B) clearly distinguish between samples under acid stress and the control. The first principal component (PC1) and second principal component (PC2) represented 36.63% and 28.41%, respectively, of the PCA in positive ion mode (Figure 4A). The first principal component (PC1) and second principal component (PC2) accounted for 41.61% and 27.17%, respectively, of the PCA in negative ion mode (Figure 4B). In addition, the 200 response sorting tests for the PLS-DA models ($R^2X$ = 0.944, $R^2Y$ = 0.999, $Q^2$ = 0.998, positive ion mode; $R^2X$ = 1.000, $R^2Y$ = 0.999, $Q^2$ = 0.856, negative ion) indicated that the models have high goodness of fit (Figure 4C, D).

These metabolites include exogenous and endogenous small molecules based on the METLIN and HMDB databases. Potential biomarkers were selected based on the biomarker criteria, using VIP > 1.0, $P$ < 0.05, and mass diff (ppm) ≤ 2 ppm. Metabolomic analysis identified changes in 16 metabolites associated with the TCA cycle, glycolysis and amino acids (Table 4). Tartaric acid and glutamate decreased significantly in both $Cd_0$ and $Cd_{100}$ soils under acid stress (pH 4.0), but fructose and lysine increased significantly in both $Cd_0$ and $Cd_{100}$ soils under acid stress. Citric acid and succinic acid were found in both the acid stress (pH 4.0) and control (pH 6.0) groups, and their contents increased significantly in $Cd_0$ soil under acid stress but decreased significantly in $Cd_{100}$ soil. Aconitic acid, oxalic acid and glucose were present in the acid stress and control treatments. Their contents decreased significantly in $Cd_0$ soil under acid stress but increased significantly in $Cd_{100}$ soil.

Table 3. Correlation analysis of factors influencing the cadmium (Cd) content in the roots of *Lolium perenne*

| | Root Cd content | Root length | Root volume | Root biomass | Root activity | Root relative conductivity | Root MDA | Root SOD activity | Root CAT activity | Root POD activity |
|---|---|---|---|---|---|---|---|---|---|---|
| Root Cd content | 1.00 | | | | | | | | | |
| Root length | 0.90** | 1.00 | | | | | | | | |
| Root volume | 0.85** | 0.98** | 1.00 | | | | | | | |
| Root biomass | 0.85** | 0.97** | 0.96** | 1.00 | | | | | | |
| Root activity | –0.02 | 0.02 | –0.11 | 0.06 | 1.00 | | | | | |
| Root relative conductivity | –0.31 | –0.20 | –0.26 | –0.21 | 0.13 | 1.00 | | | | |
| Root MDA | –0.41 | –0.57 | –0.59 | –0.63* | –0.35 | 0.50 | 1.00 | | | |
| Root SOD activity | –0.07 | 0.08 | 0.12 | 0.14 | –0.45 | 0.17 | 0.11 | 1.00 | | |
| Root CAT activity | –0.95** | –0.87** | –0.83** | –0.83** | 0.18 | 0.12 | 0.29 | –0.14 | 1.00 | |
| Root POD activity | –0.54 | –0.64* | –0.67* | –0.64* | 0.34 | 0.05 | 0.00 | –0.29 | 0.56 | 1.00 |

*$P$ < 0.05; **$P$ < 0.01; MDA – malondialdehyde; SOD – superoxide dismutase; CAT – catalase; POD – peroxidase

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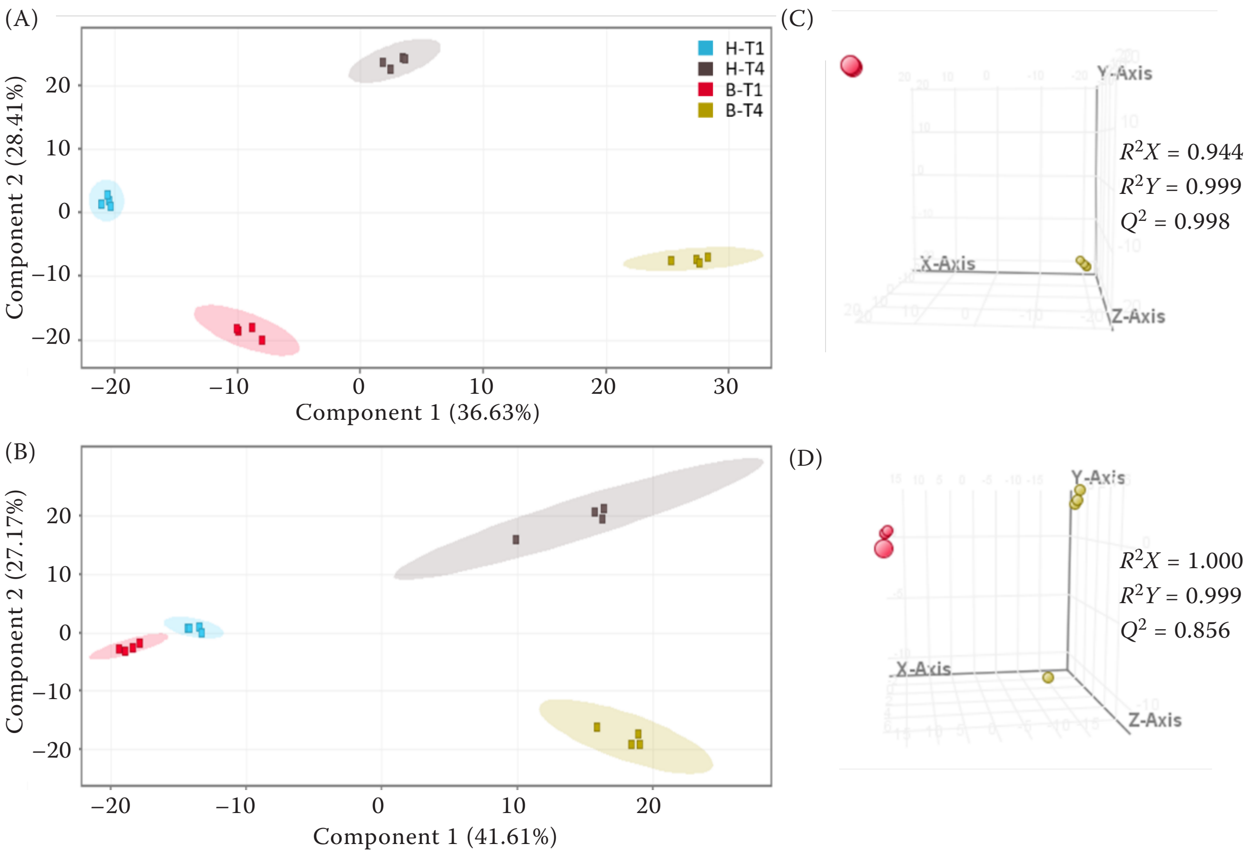


Figure 4. Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (PLS-DA) of metabolic profiles in roots of *Lolium perenne* under acid stress (four biological replicates). (A) PCA in positive mode; (B) PCA in negative ion mode; (C) PLS-DA in positive mode, and (D) PLS-DA in negative ion mode. T1 – pH 4.0; T4 – pH 6.0; H – $Cd_0$ (0 mg Cd/kg); B – $Cd_{100}$ (100 mg Cd/kg)

## DISCUSSION

**Effects of acid on the root growth of *L. perenne*.** Root growth has certain requirements for soil pH, and the effect of acid stress on root growth can be directly expressed by morphological indices such as root length, root volume and root biomass (Capó et al. 2021, Ralmi et al. 2021, Zhu et al. 2021). In this study, the root length, root volume and root biomass of *L. perenne* were significantly inhibited under high soil acid stress (pH 4.0), which may indicate that a plant's ability to absorb nutrients decreases under high soil acid stress. Root length is an important parameter used to describe the ability of roots to absorb water and nutrients (Zhu et al. 2021). *Melastoma malabathricum* L. exhibits a progressive decrease in root length as soil pH decreases (Dorairaj et al. 2020), which is consistent with the results of this study. In contrast, *Syzygium campanulatum* Korth consistently yielded high root lengths under different soil pH values (Dorairaj et al. 2020). This discrepancy in results may be due to the sensitivities of different plants to acid stress. Root volume is an important indicator for root growth and development (Ralmi et al. 2021). The results showed that in $Cd_{100}$ soil, the root volume of *L. perenne* did not differ between soil pH 4.5, 5.0 and 6.0. This indicates that the root system exhibited strong tolerance in acidic and Cd-contaminated soil. Biomass is an indicator of plant tolerance (Capó et al. 2021). In this study, the root biomass of *L. perenne* did not differ and grew well under soil pH 4.5, 5.0 and 6.0. Based on these results, it has a strong tolerance to soil acid stress. We also found that the root length, volume, and biomass in $Cd_{100}$ soil were significantly higher than those in $Cd_0$ soil under any acid stress (except root length at soil pH 4.0). Therefore, a Cd concentration of 100 mg/kg positively influenced the root growth of *L. perenne*. Wu et al. (2018) also found that Cd promoted the growth of *Abelmoschus manihot* (L.) Medik in the treatment group, even at a Cd concentration of 100 mg/kg. Similar to our result, the roots of *Medicago*

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Table 4. Relative concentration and fold changes in major metabolites in the roots of *Lolium perenne* in response to acid stress

| Metabolites pathways | Metabolites name | Relative concentration | | | | Fold changes | |
|---|---|---|---|---|---|---|---|
| | | $Cd_0$ | | $Cd_{100}$ | | $log_2$ (pH 4.0/pH 6.0) | |
| | | pH 6.0 | pH 4.0 | pH 6.0 | pH 4.0 | $Cd_0$ | $Cd_{100}$ |
| TCA cycle | citric acid | 0.00 ± 0.00 | 497.16 ± 12.16 | 1.10 ± 0.02 | 0.91 ± 0.03 | 18.07* | –0.28* |
| | aconitic acid | 1.67 ± 0.12 | 0.55 ± 0.08 | 0.59 ± 0.05 | 1.70 ± 0.10 | –1.60* | 1.53* |
| | tartaric acid | 1.27 ± 0.06 | 0.80 ± 0.02 | 1.48 ± 0.05 | 0.69 ± 0.02 | –0.66* | –1.10* |
| | malic acid | 1.17 ± 0.09 | 0.83 ± 0.10 | 0.96 ± 0.07 | 1.15 ± 0.15 | –0.51* | 0.25 |
| | succinic acid | 0.00 ± 0.00 | 927.76 ± 37.98 | 1.10 ± 0.02 | 0.91 ± 0.03 | 19.84* | –0.28* |
| | oxalic acid | 1.23 ± 0.07 | 0.86 ± 0.01 | 0.91 ± 0.05 | 1.08 ± 0.03 | –0.52* | 0.25* |
| | acetic acid | 1.69 ± 0.13 | 0.59 ± 0.06 | 1.04 ± 0.06 | 0.95 ± 0.06 | –1.53* | –0.13 |
| Glycolysis | glucose | 1.39 ± 0.07 | 0.75 ± 0.02 | 0.00 ± 0.00 | 402.29 ± 83.58 | –0.88* | 16.65* |
| | glucose-6-phosphate | 1.19 ± 0.06 | 0.83 ± 0.12 | 2.38 ± 0.90 | 0.91 ± 0.06 | –0.52* | –1.39 |
| | fructose | 0.39 ± 0.06 | 1.84 ± 0.15 | 0.49 ± 0.17 | 1.43 ± 0.02 | 2.26* | 1.56* |
| | fructose-6-phosphate | 1.21 ± 0.04 | 0.81 ± 0.12 | 0.88 ± 0.11 | 1.32 ± 0.19 | –0.58* | 0.59 |
| | sucrose | 0.31 ± 0.03 | 4.32 ± 1.27 | 0.52 ± 0.16 | 2.19 ± 0.63 | 3.79 | 2.09* |
| Amino acid | proline | 1.33 ± 0.16 | 0.88 ± 0.03 | 1.56 ± 0.17 | 0.59 ± 0.14 | –0.60 | –1.42* |
| | glutamate | 1.72 ± 0.29 | 0.61 ± 0.04 | 1.09 ± 0.08 | 0.79 ± 0.08 | –1.50* | –0.47* |
| | aspartic acid | 1.17 ± 0.09 | 0.83 ± 0.10 | 0.98 ± 0.01 | 1.02 ± 0.02 | –0.51* | 0.06 |
| | lysine | 0.42 ± 0.05 | 2.37 ± 0.26 | 0.69 ± 0.04 | 1.48 ± 0.16 | 2.51* | 1.10* |

The relative concentration of each metabolite is an average of data from four biological replicates obtained through LC-MS. Fold changes were calculated using the formula $log_2$(pH 4.0/ 6.0). *$P < 0.05$; $Cd_0$ – 0 mg Cd/kg; $Cd_{100}$ – 100 mg Cd/kg

*sativa* L. increased by about 70% in response to a Cd concentration of 50 mg/kg compared to the control group (Yang et al. 2019). In an appropriate concentration range, some plants have been shown to store toxic Cd within metabolically inactive areas such as vacuoles and the cell wall (Huguet et al. 2012), which may enable *L. perenne* to grow without any detrimental effects.

**Effects of acid on the root physiological resistance of *L. perenne*.** Root activity directly affects the growth and development of plants (Cseresnyés et al. 2020) and gives some insight into the tolerance of *L. perenne* to soil acid stress. The root activity of *L. perenne* was the lowest at pH 4.0 and significantly increased with greater soil pH. This reveals that high soil acid stress inhibits the root activity of *L. perenne*. Zhao et al. (2021) reported similar results in *Zea mays* L. Lower relative conductivity indicates less damage to the plant (Li et al. 2019). The root relative conductivity of *L. perenne* was lowest at soil pH 5.0. This is not surprising given that root length, volume, and biomass were highest at soil pH 5.0. In other words, the best growth performance aligned with the minimum value for conductivity. MDA content reflects damage to the plant somatic membranes (Bao et al. 2020). It was previously reported that low pH increased the root MDA content of *Citrus sinensis* L. (Yang et al. 2021). In our study, the root MDA content of *L. perenne* was the highest at a soil pH of 4.0 and decreased significantly with the increase in soil pH. These results indicate that high soil acid stress can produce a large amount of MDA in plant roots, seriously damaging the cell membrane and affecting the normal growth of plants. The sharp decline of root CAT and POD activities with increasing soil pH was consistent with the root MDA content of *L. perenne*. This demonstrates that plant roots can alleviate the damage of acid stress to plant cells by improving antioxidant enzyme activity, which is consistent with the research results of Wang et al. (2020).

**Effects of acid on the root Cd accumulation of *L. perenne*.** Cd accumulation by plants is one of the important indicators for screening plants in the remediation of Cd-contaminated soil (Ramana et al. 2021) and is affected by soil pH (Tian et al. 2021). Serious soil acidification affects the growth of plant roots (Figure 3), and changes in root morphology will, in turn, affect the uptake of Cd by

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roots. Furthermore, these outcomes then affect the transport and accumulation of Cd in plants (Dai et al. 2020). In our study, the root Cd content of *L. perenne* under different soil acid stress conditions (pH 4.0–6.0) was greater than 370 mg/kg. It indicated that the plant could still accumulate a significant amount of Cd under soil acid stress. In this study, the root Cd content of *L. perenne* was significantly and positively correlated with root length, root volume and root biomass. Liang et al. (2017) also observed that the Cd content in grain was positively correlated with root length and volume. Moreover, the root Cd content of *L. perenne* was negatively correlated with root CAT activity, similar to the result of *Cosmos bipinnata* (Cav.) (Huang et al. 2017). The enrichment coefficient is used as an important index to evaluate the Cd accumulation ability of plants (Ramana et al. 2021). The higher the enrichment coefficient, the stronger the Cd absorption and accumulation ability of plants is, and the more efficient it is for remediating Cd-contaminated soil (He et al. 2018, Ramana et al. 2021). The results of this study revealed that the root Cd enrichment coefficients of *L. perenne* were greater than 1 under different soil acid stress conditions, indicating that the plant roots maintained a strong ability to accumulate Cd under soil acid stress. In addition, we found that there seems to be an optimum soil pH level for Cd content and enrichment coefficient in the roots of *L. perenne*. The values were higher at pH 4.5 and 5.0 than at pH 4.0 and 6.0. Consistent with our findings, Dai et al. (2020) reported that Cd concentrations in *Bidens pilosa* L. were higher at pH 4.83 than at pH 6.81 and 7.84. Tian et al. (2021) also found that the stem Cd content of rapeseed decreased with increasing pH, and levels at pH 5.0 were significantly higher than at 6.0, 7.0, and 8.0 by 21, 24, and 40%, respectively. These results indicate that moderate soil acidity is more favourable for the Cd accumulation of some plants. The Cd content and enrichment coefficient in the roots of *L. perenne* were significantly higher than those in the shoots (Figure 5). This lead to a Cd transfer coefficient less than 1, and also indicated that the Cd absorbed by the plant was primarily enriched in the roots, consistent with the results of other studies (Zhang et al. 2019a, b). In this study, the Cd transfer coefficient of *L. perenne* decreased significantly with the increase in soil pH. The reason is primarily because increased soil pH reduces the soil available Cd and subsequently the concentrations of Cd in plants (Li et al. 2021).

**Effects of acid on the root metabolites of *L. perenne*.** Metabolomics is an important platform for studying plant stress (Feng et al. 2021). Acid stress affects root functions upon exposure; however, several organic molecules play important roles during osmotic adjustment. These include amino acids, sugars and organic acids, which potentially aid in balancing the osmotic potential of the vacuoles (Guo et al. 2018). The metabolomic results revealed that acid stress causes significant metabolic changes in the roots of *L. perenne*, including changes in the TCA cycle, glycolysis and amino acid metabolism (Table 4). Some organic and amino acids significantly decreased under acid stress (pH 4.0) in $Cd_0$ and $Cd_{100}$ soils; these included tartaric acid and glutamate. Similar findings have shown that glutamate is a compatible solute in response to drought (Guo et al. 2018) and salt stress (Jiao et al. 2018). Under acid stress, the metabolites that exhibited a significant increase were fructose and lysine. Sugar is the primary energy substance in plant metabolism (Jia et al. 2020). Our results indicated that sugar production is enhanced by acid stress. Lysine protects plant cell membranes and proteins and functions as a scavenger of reactive oxygen species (Ali et al. 2022). In our study, the active synthetic metabolism of nutrients, including fructose and lysine, was dramatically enhanced in roots, which improved ROS detoxification capacity, membrane stability and acid tolerance (Guo et al. 2018, Jiao et al. 2018). Meanwhile,

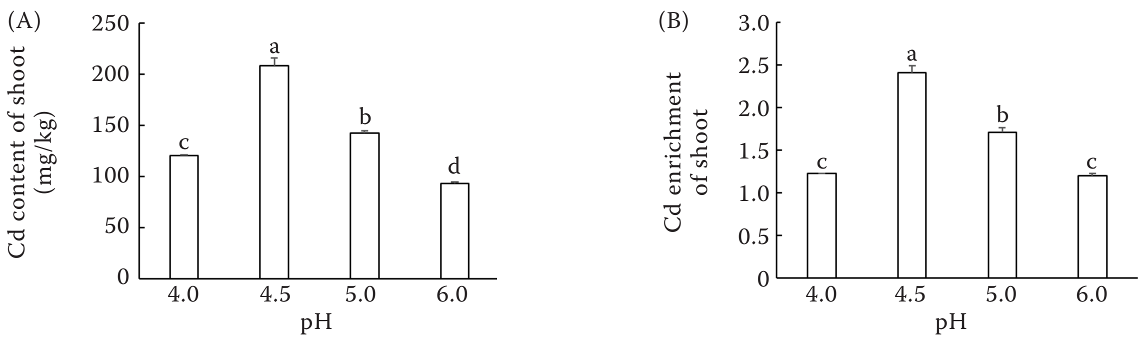


Figure 5. Effects of acid on the shoot cadmium (Cd) accumulation of *Lolium perenne* in $Cd_{100}$ (100 mg Cd/kg) soil

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in response to acid stress, the citric acid and succinic acid contents of roots in $Cd_0$ soil increased significantly, whereas their contents decreased significantly in $Cd_{100}$ soil. Furthermore, aconitic acid, oxalic acid and glucose decreased significantly in $Cd_0$ soil under acid stress but increased significantly in $Cd_{100}$ soil. This indicates that Cd concentration also affects root organic acids and glycolysis, which requires further studied (Yan et al. 2020). In plants, organic acids (citric, aconitic, tartaric, succinic and oxalic), sugars (glucose and fructose) and amino acids (glutamate and lysine) have been shown to be important for improving plant stress resistance (Guo et al. 2018, Jiao et al. 2018, Jia et al. 2020). We suggest that the most important compatible solutes are tartaric acid, fructose and amino acids (glutamate and lysine) in the roots of *L. perenne* under acid stress.

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