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The Exogenous Application of Malic Acid Eliminates Cadmium Concentrations in Aromatic Rice of Pulu Mandoti Emas Variety

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Abstract
Food security and human health are at risk due to rice fields' heavy metal cadmium (Cd) pollution, which has become a global problem. As rice is a significant crop in Indonesia, reducing the detrimental effects of Cd accumulation in rice plants requires serious consideration. This study aimed to evaluate the effect of malic acid on the availability and uptake of Cd (in soil, roots, and shoots), the relative water content (RWC), the morphological growth, and the anatomical structure of roots in the local aromatic rice of the Pulu Mandoti Emas (PME) variety. The concentrations of Cd ($CdCl_2$), comprising control (C1) and 10 mg/kg Cd (C2), and malic acid ($C_4H_6O_5$), which included control (M1) and 50 µM malic acid (C2), were employed in this study. The results showed that Cd was only detected in the C2M1 at levels of 0.05 ppm in the soil and 4.47 ppm in the roots. The C2M2 eliminated Cd contents in the soil, roots, and shoots and further showed the highest values for the RWC (69%), root length (12.51 cm), and root fresh weight (4.74 g) compared to other treatments. Anatomically, the C2M2 maintained the structural integrity of the root tissue, while the C2M1 disrupted the cortex tissue. The use of malic acid is recommended to preserve water potential and plant growth, as well as protect root anatomy under Cd exposure.

Keywords: Cd-contaminated paddy; heavy metal; organic acid

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INTRODUCTION

Rice is a staple food worldwide that is often reported to be contaminated by heavy metals. One is a non-essential metal of cadmium (Cd) with persistent characteristics, mobility, and binding to soil colloidal complexes or other mineral elements, leading to high bioavailability for root absorption (Z. Li et al., 2021). Cd accumulation in cereal crops is a global environmental and health concern (Hussain et al. 2021). Cd pollution in some agricultural areas in Indonesia is caused by industrial waste and household products, domestic and agricultural waste, mining, and cultivation inputs and is exacerbated by the repeated use of agrochemicals such as fertilizers and pesticides (Sutrisno & Kuntyastuti, 2015; Meriem, 2023). Some polluted agricultural lands in Indonesia contain Cd in the 0.10 to 9.60 ppm range. Meanwhile, the maximum limit of Cd content in rice is 0.4 mg/kg (SNI 7387:2009) according to the Badan Standardisasi Nasional (2009).

Plants absorb and accumulate Cd in their various organs and tissues. Cd accumulated in rice grains is upstream of Cd transport by roots, root-shoot translocation through xylem flow, node translocation, and leaf part remobilization mediated by several specific transporter proteins (Uraguchi and Fujiwara, 2012). Morphological and anatomical root structures play an important role in Cd uptake and translocation in rice plants, including genotypes and rice growth conditions (Huang et al., 2019). The consumption of Cd-containing rice accounts for a higher carcinogenic risk than other heavy metals (Fan et al., 2017; Shao et al., 2023) and causes respiratory, nephrological, circulatory, skeletal, reproductive, and nervous system disorders in humans (Charkiewicz et al., 2023).

Consumption of Cd-contaminated rice threatens human health. Therefore, it is necessary to consider practical strategies to reduce Cd contamination and accumulation in rice grains. Remediation methods using organic fertilizer amendments have been reported to increase crop yields in polluted farmlands and reduce Cd levels in rice grains (Zeng et al. 2019). In addition, microbial-based amendments, such as vesicular arbuscular mycorrhiza, reduce the Cd content in plant tissues (Nurlaili et al., 2021). Spraying 400 mL/L of humic acid increases plant growth and productivity (Rahim et al., 2022). Humic acid not only increases rice yield but can also inhibit the translocation and remobilization of Cd towards the canopy during grain filling and increase Cd immobilization through carboxyl, hydroxyl, and amino acid bonds in the cell wall (Deng et al., 2022). Organic acids from malate compounds inhibit Cd accumulation in rice roots and the canopy by increasing glutathione, aspartic acid, and glutamate levels through malic acid-aspartic acid metabolic transformation (Zhang et al., 2023). Malic acid supplementation in media contaminated with Cd significantly reduced Cd phytotoxicity and its accumulation in plant tissue due to the ability of this acid to lower residual Cd and exchangeable Cd, compared with other low molecular weight organic acids such as oxalic acid, acetic acid, and citric acid (Lu et al., 2021). Moreover, exogenous malic acid exhibited the greatest effect in increasing plant biomass and reducing Cd concentration and lipid peroxidation in the roots compared to citric acid and tartaric acid (Zhang et al., 2020). The complexation of organic acids with Cd depends on the crop and different organic acids. Considering the superiority of malic acid, the evaluation should consider different species.

The potential for Cd to be detected in aromatic local rice varieties of Pulu Mandoti Emas (PME) is increasing because of the transition to the use of manure combined with NPK chemical fertilizers in rice fields repeatedly over a long period of time. Moreover, the planting area of this variety is limited to the rice fields of Salukanan Village, Enrekang Regency. Therefore, there is a need for early mitigation efforts to protect local PME rice from Cd contamination. This study aimed to evaluate the effect of exogenous application of malic acid on Cd availability and uptake, morphological growth, relative water content, and the anatomical structure of PME rice.

MATERIALS AND METHODS

This study used a completely randomized design (CRD) with two factors: (1) cadmium treatment and (2) malic acid treatment. The cadmium ($CdCl_2$) treatment consisted of two concentration levels, namely control (0 mg/kg Cd) and 10 mg/kg Cd, whereas the malic acid ($C_4H_6O_5$) treatment consisted of two concentrations, namely control (0 µM malic acid) and 50 µM malic acid. There were four treatments, each with three replicates.

PME rice seeds were collected from farmers in Salukanan Village, Baraka District, Enrekang Regency, South Sulawesi. The seeds were soaked in water for 24 h and germinated for 2 d in the dark. The germinated seeds were sown in planting media consisting of soil and compost at a ratio of 1:1 for 20 days. Twenty days after sowing, the rice seeds (HST) were transplanted into polybag planting media and grown for 30 days at the vegetative stage. Fertilization (urea, SP-36, and KCl) was administered twice, at 10 DAP and 40 DAP. Treatments were administered after 50 DAP and observed for 14 days.

Root length (cm), root wet weight (g), root dry weight (g), shoot fresh weight (g), and shoot dry weight (g) were measured for morphological parameters. Shoot and root dry weights were measured after drying at 80 °C until they reached a constant weight. The root anatomy was determined using the transverse sectioning technique. The root incision was placed on an object glass, stained with 0.1% methylene blue, and observed under a light microscope.

Ten fully developed rice leaves were drilled with a 0.5 cm diameter cork borer and weighed to obtain fresh weight (FW). The leaf samples were soaked in distilled water for 24 h and weighed to obtain the saturated weight (SW). Next, the samples were dried at 60˚C in an oven until they reached a constant weight to obtain the dry weight (DW). The relative water content (RWC) value was calculated using the following equation (Meriem et al., 2021):

$$Relative\ Water\ Content\ (RWC) = \frac{FW\ (g) - DW\ (g)}{SW\ (g) - DW\ (g)} \times 100$$

Each treatment combination's soil, root, and crown samples were taken as a composite for cadmium (Cd) content testing using an Atomic Absorption Spectrophotometer (AAS) based on the SNI 8910:2021 method. The plant samples were then crushed using a mortar and pestle. A total of 5 g of the sample was added to 10 mL of HNO_3 (1:1; concentrated HNO_3: distilled water), homogenized, heated at 95 ˚C for 10 min, and cooled. The sample solution was added to 5 mL of HNO_3 p.a., the heating was repeated to a volume of 5 mL, and the solution was cooled. The test solution was added to 2 mL demineralized water, 3 mL 30% H_2O_2, and 1 mL H_2O_2 aliquots to boiling, and boiled until the volume dropped to 5 mL. The test solution was prepared by adding 10 mL of HCl p.a., heated for 5 min, filtered, and the Cd content was measured using AAS at a wavelength of 228.8 nm.

The data were analyzed by one-way ANOVA using SPSS Ver.25. Multiple comparisons were analyzed using Tukey's test to compare statistically significant differences between the groups. All analyses were performed at α=5%.

RESULTS

Under Cd exposure without malic acid (C2M1) treatment, Cd was only positively detected in the soil and was highly accumulated in the roots. The application of malic acid (C2M2) resulted in the loss of Cd in the soil and thus removed its contents both in the roots and shoots (Table 1). This finding was followed by the highest relative water content (RWC) indicated for C2M2 among the other treatments (Figure 1). The lowest RWC was observed for C2M1.

Table 1. Cd concentrations in soil, roots, and shoots of the PME variety.

Treatments	Cd (ppm)		
	Soil	**Root**	**Shoot**
C1M1 (0 mg/kg Cd + 0 µM malic acid)	nd	nd	nd
C1M2 (0 mg/kg Cd + 50 µM malic acid)	nd	nd	nd
C2M1 (10 mg/kg Cd + 0 µM malic acid)	0.05	4.47	nd
C2M2 (10 mg/kg Cd + 50 µM malic acid)	nd	nd	nd

Note: nd (not detected)

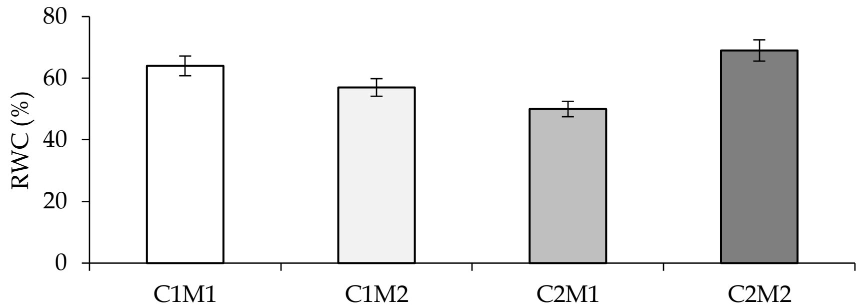


Figure 1. Relative water content (RWC) of PME leaves in each treatment. C1M1 (0 mg/kg Cd +0 µM malic acid), C1M2 (0 mg/kg Cd +50 µM malic acid), C2M1 (10 mg/kg Cd +0 µM malic acid), dan C2M2 (10 mg/kg Cd +50 µM malic acid)

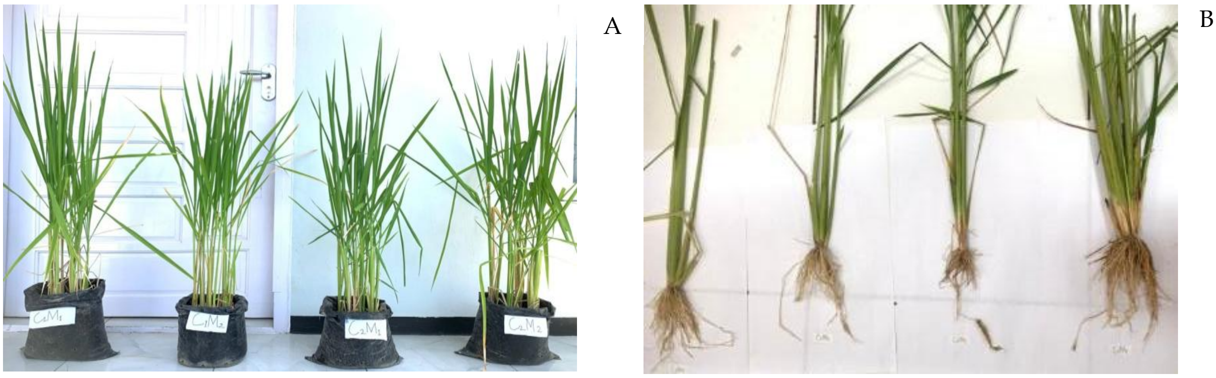
The treatments did not differ significantly in terms of morphological parameters (Table 2). Based on the mean value, it can be seen that Cd exposure in the C2M1 reduced the length and dry weight of roots as well as the fresh weight and dry weight of shoots. Under the same conditions, malic acid applied to C2M2 resulted in a high mean root length, fresh weight of roots, and dry weight of shoots. The performance of the PME plant is shown in Figure 2.

Table 2. Effects of treatments on morphological characteristics of the PME variety.

Treatments	Root length (cm)	Root (g)		Shoot (g)	
		Fresh weight	**Dry weight**	**Fresh weight**	**Dry weight**
C1M1 (0 mg/kg Cd + 0 µM malic acid)	11.67±0.88	4.64±1.80	0.28±0.05	34.61±2.16	4.15±0.34
C1M2 (0 mg/kg Cd + 50 µM malic acid)	10.00±1.15	4.24±1.62	0.26±0.09	34.69±2.91	4.14±0.10
C2M1 (10 mg/kg Cd + 0 µM malic acid)	8.50±0.29	4.50±1.32	0.25±0.07	23.88±5.47	3.42±0.09
C2M2 (10 mg/kg Cd + 50 µM malic acid)	12.51±1.78	4.74±1.89	0.27±0.08	29.92±5.35	3.49±0.12

Note: Value showed Mean±SE

The anatomical structure of the root PME is shown in Figure 3. Compared with the control treatment (C1M1), Cd stress disintegrated the cortex layers in the C2M1 treatment. This section also indicated that the application of malic acid under Cd exposure (C2M2) did not cause root damage.



C1M1 C1M2 C2M1 C2M2 C1M1 C1M2 C2M1 C2M2

Figure 2. The performance of shoots (A) and roots (B) of the PME variety under the treatments. C1M1 (0 mg/kg Cd +0 µM malic acid), C1M2 (0 mg/kg Cd +50 µM malic acid), C2M1 (10 mg/kg Cd +0 µM malic acid), dan C2M2 (10 mg/kg Cd +50 µM malic acid)

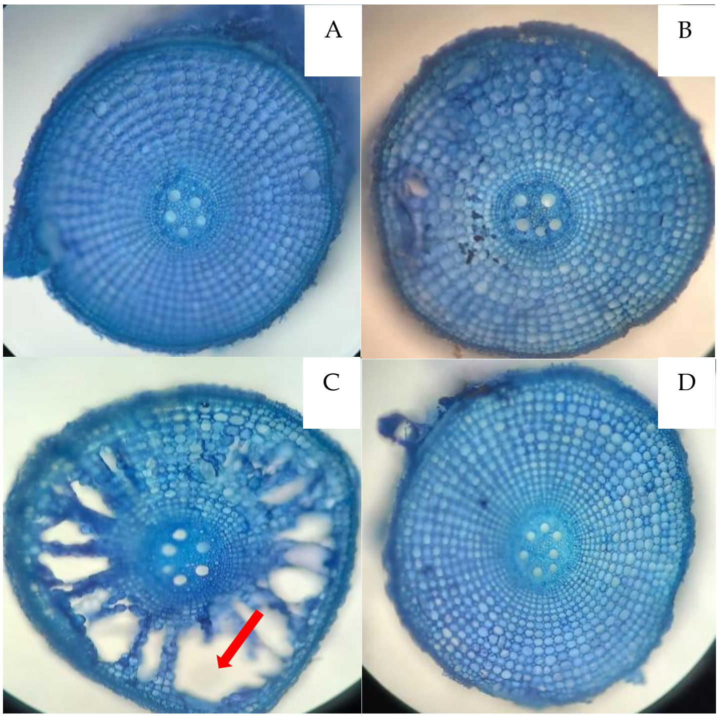


Figure 3. Root anatomy of the PME variety under different treatments. (A) C1M1 (0 mg/kg Cd +0 µM malic acid), (B) C1M2 (0 mg/kg Cd +50 µM malic acid), (C) C2M1 (10 mg/kg Cd +0 µM malic acid), and (D) C2M2 (10 mg/kg Cd +50 µM malic acid). The red arrow indicates the disintegrated root cortex tissue. Magnification 4x10

DISCUSSION

The process of Cd chelation by malic acid can be explained by monitoring and measuring the Cd content in the soil, roots, and shoots of the PME variety in each treatment. Table 1 shows that under 10 mg/kg Cd stress, the application of 50 µM malic acid (C2M2) eliminated the availability of Cd in the soil so that neither Cd was absorbed by the roots nor translocated to the shoots. This finding is supported by Meriem (2023), who stated that malic acid is a chelator from the organic acid group that binds two Cd^{2+} ions to its carboxyl group. The malic acid chelation process of Cd limits Cd migration to the shoots by increasing root activity (Hawrylak-Nowak et al., 2015) and assists in the Cd exclusion mechanism.

Under Cd-stress conditions without malic acid (C2M1), Cd was detected in the soil at a concentration of 0.05 ppm. Without malic acid, Cd became available in the soil, allowing the roots to absorb Cd in a larger amount of 4.47 ppm. This concentration has exceeded the maximum threshold of Cd content in rice plants, which is 0.4 mg/kg (Badan Standardisasi Nasional, 2009). The level of 4.47 ppm detected in the roots in our study was found to be higher than the concentration of Cd in the roots of rice plants in the studies of (Juen et al., 2014), (Satpathy et al., 2014), and (Payus et al., 2015) as much as 0.29, 0.20, and 0.38 mg/kg, respectively. However, the shoots in the C2M1 treatment did not show the amount of Cd. We assumed that (1) the process of Cd compartmentalization in the roots of PME rice and (2) the short period of 14 days of investigation resulted in the translocation process not being detected in the shoot area. The high concentration of Cd in the roots is supported by Zakaria et al. (2021), who explained that the accumulation of heavy metals is mainly found in the roots compared to the shoots and grains. The rate of Cd translocation is influenced by rice genotypes, such as the rice cultivar JYT36, which has a high Cd uptake and accumulation ability in the roots but a low shoot translocation rate because this cultivar has a Casparian strip and a thick suberin layer (Anwen et al., 2021). Nevertheless, this requires further investigation.

In this study, cadmium (Cd) stress reduced the Relative Water Content (RWC) and inhibited the growth of PME plants. In contrast, the addition of malic acid under Cd stress conditions enhanced RWC and maintained root growth parameters. The highest RWC value of 69% was observed under Cd stress conditions after treatment with malic acid (C2M2), while the Cd treatment without malic acid (C2M1) showed the lowest RWC (50%) (Table 2). Physiologically, RWC that can be maintained under Cd stress conditions allows plants to stabilize metabolic processes that balance plant growth and development. This condition allowed plants to increase their root length and root biomass in the C2M2 treatment (Table 2). Compared to the other treatments, C2M1 treatment showed the lowest growth parameters of root length, root dry weight, and shoot dry weight (Table 2). Several studies have also reported a decrease in root and shoot biomass after Cd application compared to the control treatment in rice plants (Guo et al., 2017; Ronzan et al., 2018).

The mechanism of Cd ion binding by malic acid prevents Cd availability in soil. Therefore, plant roots are prevented from absorbing Cd; hence, tissue damage and physiological effects of Cd can be avoided. Addition of malic acid under Cd stress conditions has also been reported to increase total biomass and growth rate by increasing enzymatic antioxidant activity and enhancing photosynthetic pigments (Vega et al., 2022). Malic acid confers plant resistance to Cd-induced oxidative stress, reduced lipid peroxidation, increased glutathione content, and phytocellulin biosynthesis (Zhang et al., 2020). This organic acid blocked Cd uptake through the root exclusion process, increased OsHMA3 transporter protein, which plays a role in triggering Cd sequestration in the roots, and increased the accumulation of antioxidants such as anthocyanins and glutathione under Cd stress conditions (Sebastian & Prasad, 2018).

Regarding morphology, PME plants in the Cd with malic acid treatment (C2M2) exhibited favorable root growth. Figure 1 shows that the same number of rice clumps produced a higher root volume and length in the C2M2 treatment than in the other treatments. This finding indicates that in addition to binding Cd in the soil, malic acid can shield the roots of PME rice from Cd injury, thereby increasing root growth. In contrast, Cd stress in the absence of malic acid in C2M1 inhibited root volume and length. This condition can be explained by the uptake of Cd by the roots in high amounts (4.47 ppm), which decreased the KAR value and inhibited PME rice plant growth. Similar results were reported by Ronzan et al. (2018), who found that the application of 100 μM CdSO4 reduced adventitious root length, lateral root primordia density, and lateral root density by 50% compared with the control treatment.

To support the data from the morphological observations, we performed anatomical sections of the roots to examine the impact of Cd stress on root tissue structure. Figure 2 shows the deterioration of root tissues in the Cd treatment without malic acid (C2M1). High Cd uptake in the roots after C2M1 treatment at 4.47 ppm (Table 1) caused severe destruction of the parenchyma tissue that made up the root cortex layer, which was characterized by the formation of cavities (Figure 2c). Meanwhile, with the application of malic acid, Cd removed by malic acid in the soil (C2M2) showed a clear root anatomy and exhibited a structure similar to that of the control (C1M1) and C1M2 treatments. The damaged cortical tissue in C2M1 was probably caused by high Cd accumulation.

A damaged cortex layer causes plant roots to be unable to absorb water and minerals from the epidermis to the endodermis and xylem transport tissue through the symplastic or apoplast pathway. At the same Cd concentration (10 mg/kg), the root anatomy of the rice cultivar TLY215 also showed intercellular tissue damage and an increase in the number of channels in the cortex layer, which is characterized by cell tissue spaces (Lan et al., 2023). Another study by Li et al. (2016) also showed that 3.0 Cd mg/L caused damage to the cortex and epidermis tissue in rice, followed by the formation of aerenchyma in the cortex layer and shrinkage of the xylem tract. Cadmium at a concentration of 100 μM Cd induces damage to the formation of lateral root primordia in pericycle and endodermis cells, which causes disorientation or anomalous shape of rice tissue, as well as high accumulation of Cd in the cortex tissue, resulting in cell plasmolysis (Ronzan et al., 2018). Cadmium also induces premature formation of aerenchyma differentiation in the lateral root primordia tissue. Similar to the findings of this study, the cortex is the main tissue area that accumulates the most heavy metals via the apoplast pathway compared to pericycle tissue and endodermis (Seregin et al., 2004; Rebouillat et al., 2009). We suspected a more destructive invasion of anatomical damage in the stele tissue area (endodermis, pericycle, phloem, xylem, and pith) at high exposure durations. Structural changes in root tissue may be one of the main causes of the decline in plant biomass in this study.

Based on these findings, malic acid is promising for sustainable agricultural practices that reduce the impact of Cd deposited in soils or remediate idle land caused by accumulated heavy metals. However, further studies are needed to optimize the malic acid concentration in cross-species and Cd conditions, root system response, long-term effects of malic acid, and its combination with other phytoremediation strategies.

CONCLUSION

The addition of 50 μM malic acid under stress conditions of 10 mg/kg Cd (C2M2) eliminated the Cd content in the soil, roots, and shoots of the Pulu Mandoti Emas (PME) rice variety, increased the RWC and root biomass, and maintained the integrity of the root anatomical structure. Cd stress treatment without malic acid (C2M1) accumulated Cd in the root area, decreased RWC, and inhibited root and shoot biomass; and (3) damaged the cortical tissue. Malic acid supplementation can be applied in agricultural practices to alleviate soil-affected Cd levels in rice fields.

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CONFLICT OF INTEREST

There is no conflict of interest.

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