Environmental Science and Pollution Research (2023) 30:3668–3687
https://doi.org/10.1007/s11356-022-22478-y

RESEARCH ARTICLE

Physiological and metabolic dynamism in mycorrhizal and non-mycorrhizal *Oryza sativa* (var. Varsha) subjected to Zn and Cd toxicity: a comparative study

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Received: 29 December 2021 / Accepted: 7 August 2022 / Published online: 11 August 2022

Abstract

Arable lands getting contaminated with heavy metals have a very high negative impact on crop plants. The establishment of the mycorrhizal association with crop plants is a sustainable strategy to overcome metal toxicity. The major aim of this study was to analyze mycorrhizae-mediated alterations on the physiology and metabolism of *Oryza sativa*, as well as the impact of these alterations in the metal tolerance potential of the host on exposure to cadmium (Cd) and zinc (Zn) stresses. For this, 45 d old *O. sativa* (var. Varsha) plants inoculated with *Claroideoglomus claroideum* were exposed to 1.95 g Zn kg^{-1} soil and 0.45 g Cd kg^{-1} soil. Mycorrhization significantly increased shoot weight, root weight, moisture content, and chlorophyll biosynthesis under Cd and Zn stresses. Mycorrhization mitigated the oxidative stress elicited in *O. sativa* by the elevated Cd and Zn content, and it aided in maintaining the metabolite's level and rate of photosynthesis as compared to non-mycorrhizal plants. The circular-shaped unique structures seen as opening on the leaf surface of non-mycorrhizal plants under Zn stress, possibly for the emission of volatile compounds synthesized as a result of Zn stress, have a great chance of leaf tissue destruction. This structural modification was characterized in the case of Zn stress and not in Cd stress and can lead to the reduction of photosynthesis in *O. sativa* exposed to Zn stress. The reduction in oxidative stress could be correlated to the reduced uptake and transport of Cd and Zn ions in mycorrhizal plants. The exudation of tributyl acetyl citrate, 3-beta-acetoxystigmasta-4,6,22-triene, and linoleic acid from the mycorrhizal roots of rice plants has a crucial role in the stabilization of metal ions. This study proposes mycorrhization as a strategy to strengthen the Cd and Zn stress tolerance level of rice plants by regulating the physiology and metabolomics of the host plant.

Keywords Cadmium · GC-MS · Metabolites · Mycorrhiza · Rice · Zinc

Introduction

The contamination of agricultural lands with heavy metals is a growing concern of the human population owing to the biomagnification potential of these elements in the edible portion of crops. Cadmium (Cd) and zinc (Zn) contamination have been reported in different agricultural lands, especially in the paddy fields (Wang et al. 2019; Janeeshma et al. 2021a). Rice is one of the most important staple foods for half of the world's population, and its production has to be increased to meet the growing demand. Rizwan et al. found that rice cultivation in heavy metal contaminated agricultural lands invites the risk of biomagnification at different trophic levels due to the potential of rice plants to uptake and translocate these toxic metals to shoots (Rizwan et al. 2016). The further transfer of metal ions like Cd and Zn to edible portions of rice plants turns out to be critical for mankind (Uraguchi et al. 2009; Gu et al. 2012).

Cadmium is a non-essential element widely introduced to the soil by anthropogenic smelting and mining activities. The exposure of plants to high concentrations of Cd affects the growth and development of plants. Cadmium toxicity causes visible injuries like chlorosis, growth inhibition, browning of root tips, and finally leads to the death of the plant (Wojcik and Tukiendorf 2004; Janeeshma et al. 2021b). Cadmium induces the generation of reactive

Responsible Editor: Gangrong Shi

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oxygen species (ROS) and causes lipid peroxidation, indicated by a higher accumulation of malondialdehyde (Singh and Shah 2014). Cadmium also affects photosynthesis by down-regulating photosystem II activity (Singh et al. 2019; Janeeshma and Ahmad 2020). At the same time, Zn, one of the essential elements, turns toxic to plants only when its concentration exceeds the tolerance limit (Huang and Ma 2020; Janeeshma et al. 2021c). Overaccumulation of Zn in plants induces detrimental effects by causing metabolic imbalances, affecting electron migration, and decreasing membrane permeability (Rout et al. 2019; Sarraf et al. 2022). Zinc also triggers the overproduction of ROS, causing oxidative stress by the misbalance of antioxidation machinery (Cui and Zhao 2011).

The selection of low metal accumulating cultivars, crop rotation, water management, and the application of different soil types have been successfully employed to reduce the metal uptake by rice. The introduction of a microbial population with heavy metal stress tolerance potential to the rhizosphere of rice plants will increase the Cd and Zn stabilization potential (Roychoudhury and Tripathi 2020; Prakash et al. 2020). Arbuscular mycorrhiza (AM) is considered a promising candidate for achieving metal stabilization in the rhizosphere, and it prevents the translocation of metals to the above-ground portions and thus to the food chain. The association of a native mycorrhizae population with rice roots and the heavy metal stress tolerance potential of these mycorrhizae-associated plants were elaborated in different studies (Zhang et al. 2005; Li et al. 2016; Chen et al. 2019b; Janeeshma and Puthur 2020). In addition, reports in previous works pointed out the role of mycorrhizae in the immobilization of metal ions in the host rhizosphere (Ruscitti et al. 2017; Mitra et al. 2021). However, a comprehensive study on the physiological, micromorphological, and biochemical aspects of the root and shoot tissue of rice plants is necessary to understand the Cd and Zn stress tolerance mechanisms operational in rice. The present study validates the phytostabilization potential in rice through specific physiological and metabolic dynamisms adapted under Cd and Zn stresses as a result of AM association.

Materials and methods

Collection of plant material and inoculum

Rice (variety Varsha) seeds were collected from the Regional Agricultural Research Station (RARS) of Kerala Agricultural University, Pattambi, Kerala, India, and the inoculum of *Claroideoglomus claroideum* was procured from the Centre for Mycorrhizal Culture Collection (CMCC), The Energy and Resources Institute (TERI), New Delhi.

Multiplication of the inoculum

Multiplication of the spores of *C. claroideum* was carried out by the pot culture method. For this purpose, 3 kg of sterilized soil (soil:sand in 1:1) was filled in each pot. Further, the soil was inoculated with 80 spores of *C. claroideum*. Then the seeds of *Zea mays* surface-sterilized with 0.1% $HgC1_2$ (w/v) solution for 5 min were sown in these pots. When the *C. claroideum* colonization level was 90–100% (60 days after sowing), shoots of maize were cut, and the remaining root parts were uprooted. The soil in the pot with AM-associated roots was taken as the AM inoculum, which contained spores, mycelium, and root fragments.

Root colonization analysis

Root colonization of *C. claroideum* was visualized with the help of 0.01% of trypan blue staining, as per the protocol of Phillips and Hayman (1970).

Experimental design

Surface sterilized rice seeds (3 nos) were sowed with a planting depth of 8 cm in the sterilized soil filled in polythene bags (18 × 13 cm). Two sets of plants were used for the experiment, one with AM inoculation ($^{+}$M plants) and the other without AM inoculation ($^{-}$**M** plants). Twenty grams of *C. claroideum* inoculum (containing approximately 320 spores per gram) was applied to the soil for AM association. Polythene bags were maintained in a polyhouse with controlled growing conditions (60 ± 2% relative humidity, 25 ± 2 °C temperature, and 12 h daylight ranging from 700 to 900 µmol m^{-2} s^{-1}). During the initial growth stages, plants were watered with distilled water, and further 50 mL of quarter-strength modified Hoagland solution was applied (the Hoagland solution was prepared by avoiding $ZnSO_4 \cdot 7H_2O$). After 45 days of growth, 40 mL (the field capacity of the soil) solutions with 1.95 g Zn kg^{-1} soil as $ZnSO_4$ and 0.45 g Cd kg^{-1} soil as $CdCl_2$ were applied to two different sets of plants. These concentrations of $ZnSO_4$ and $CdCl_2$ were screened from different concentrations of $ZnSO_4$ (0.0, 0.65, 1.30, 1.95 g Zn kg^{-1} soil) and $CdCl_2$ (0, 0.225, 0.45, 0.675 g Cd kg^{-1} soil), and the primary screening analysis was carried out in 4-day intervals (0, 4, 8, and 12 days). Moisture content %, total chlorophyll, and MDA content were the parameters studied for selecting stress imparting concentration. The second lower leaf and the roots of the rice plants were taken for various analyses. The data is an average of three independent treatments with three replicates (i.e., $n = 9$).

Fresh weight (FW) and dry weight (DW) percentage

An electronic weighing balance was directly used to measure the fresh weight of the rice plants. After the measurement of fresh weight, the root and leaf tissues were dried at 100 °C for 1 h followed by 60 °C in a drying oven until the weight attained a constant value. These constant values were considered as the dry weight of plant tissues.

Tissue moisture content %

The tissue moisture content was measured using the following equation (Lokhande et al. 2011).

$$\text{Moisture content}\% = \left[(\text{FW} - \text{DW})/\text{FW}\right] \times 100$$

Root volume

The root volume of rice plants was measured according to the protocol of Rahul et al. (2019).

Determination of cell viability

The loss of cell viability in the roots of metal-treated plants was evaluated by the modified method of Šimonovičová et al. (2004), using Evans blue stain. Roots were stained in a 0.25% (v/v) aqueous solution of Evans blue for 15 min at room temperature. The stained roots were washed three times with distilled water for 10 min each. Root tips (5 mm) were excised and soaked in 50% (v/v) methanol along with 1% (w/v) SDS at 60 °C for 1 h. The optical density of the destained solution was measured spectrophotometrically at 600 nm.

Micromorphological characters of leaves

The micromorphology of leaves was analyzed using a scanning electron microscope (SEM). The leaf cuttings of different treatments were fixed in glutaraldehyde (2.5%) and prepared in sodium cacodylate buffer (pH 7.2) for 5 min. Fixed specimens were washed with double-distilled water and dehydrated using an acetone series. Dehydrated leaf tissues were mounted on grooves cut on aluminum stubs using conducting carbon tape. Further, the specimens were coated with gold-palladium particles, and photomicrographs of the specimens were taken using the photographic attachment of the Field Emission Scanning Electron Microscope (Carl-Zeiss-Gemini 300).

Total chlorophyll content

For the estimation of chlorophyll, the leaf tissue (0.5 g) was homogenized in 80% acetone, and the total chlorophyll content was calculated based on the method of Arnon (1949).

Chl a fluorescence parameters

Plant Efficiency Analyzer (Handy PEA, Hansatech Ltd, Norfolk, UK), a portable fluorometer was used to measure Chl *a* fluorescence transients from the adaxial leaf surface (Strasser et al. 2004). All measurements were performed after 20 min of dark adaptation, where light exclusion clips were used to restrict the exposure of light. Maximal fluorescence was induced by a 1 s pulse of continuous light (650 nm, 3,000 µmol m^{-2} s^{-1}). Chl *a* fluorescence signals were analyzed with Biolyzer HP3 software (Laboratory of Bioenergetics, University of Geneva, Switzerland). Various fluorescence parameters like the area over the curve, the photochemical efficiency of PSII (Fv/Fm), ABS/CSm (number of photons absorbed by a PS II cross-section), and electron transport per cross section (ETo/CSm) were measured in relative units (RU), and the specific membrane leaf model was deduced in response to Cd and Zn stresses in rice.

ROS accumulation

Superoxide (O_2^-) content

Two hundred milligrams of the plant tissue were cut into 1 × 1 mm size and immersed in 0.01 m potassium phosphate buffer (pH 7.8) containing 0.05% nitro blue tetrazolium (NBT) and 10 mM NaN_3 (sodium azide), and the superoxide content was estimated as described by Doke (1983).

Hydrogen peroxide content

Two hundred milligrams of tissue were homogenized in 5 ml of 0.1% ice-cold trichloroacetic acid and centrifuged at 12,000 rpm for 15 min. The supernatant was collected, and hydrogen peroxide was estimated as described by Junglee et al. (2014).

Estimation of malondialdehyde (MDA)

MDA was extracted and estimated according to the method of Arun et al. (2021). The MDA concentration was calculated using its molar extinction coefficient of 155 mM L^{-1} cm^{-1}.

Estimation of primary metabolites

Free proline content was extracted from the leaves and roots of rice plants using 3% sulfosalicylic acid and estimated using the protocol of Bates et al. (1973), where L-proline was the standard.

Based on the protocol of Dubois et al. (1956), total soluble sugar content in rice plants was estimated, where D-glucose was used as the standard.

Free amino acid content in rice plants was assessed by the protocol of Moore and Stein (1948) using ninhydrin reagent, where glycine was used as the standard.

Estimation of secondary metabolites

The total phenolic content of rice tissues was estimated based on the protocol of Folin and Denis (1915) using Folin–Ciocalteau reagent (0.5 ml of 1 N) and catechol used as the standard.

For performing the GC-MS analysis, the root and leaf tissues of different treatments were shade dried at room temperature. Extracts were prepared as per the method of Grover and Patni (2013), using methanol as the solvent. GC-MS (QP2010S, Shimadzu, Italy) with Rxi-5Sil MS column (30 m length × 0.25 mm ID × 0.25 µm thickness) for the detection of different bioactive compounds. Initially, the column temperature was 80 °C for 4 min, increased to 260 °C, and held for 6 min. Different metabolites in the methanolic extracts were identified by comparing their retention indices and mass spectra fragmentation patterns with those stored in the computer library (NIST 11 and WILEY 8).

Estimation of Zn and Cd content in plant tissues

The concentration of Zn and Cd in the dried samples was assessed as per the method of Janeeshma et al. (2021a). One gram of sample was digested by refluxing in a 5:3 ratio of nitric:perchloric acid until the solution became colorless using Kjeldahl flasks heated (60 °C) in a heating mantle. After the filtration, the filtrate was made up to 100 mL using a standard flask. An atomic absorption spectrophotometer (AA-7000, Shimadzu, Kyoto, Japan) was used to analyze Zn and Cd present in the digested samples.

Statistical analysis

Statistical analysis of the results was carried out according to Duncan's multiple range tests at a 5% probability level. Data were subjected to one-way ANOVA using the SPSS software 16.0. The data is an average from three independent experiments, each with three replicates. The data represents the mean ± standard error.

Results

Colonization rate of *C. claroideum*

The presence of different mycorrhizal structures like arbuscules, vesicles, and hyphae was determined by the colonization of *C. claroideum* in the roots of *O. sativa*. Ninety to 100% of root infection was observed in AM-associated plants at 45 days of growth (Fig. 1). The roots of $^{-}$M plants did not exhibit any mycorrhizal structures.

Root volume and cell viability

Root volume extensively increased in $^{+}$M plants related to $^{-}$M plants (Table 1). The root volume significantly decreased under Cd and Zn stress, and the decrease was to the extent of 54 and 31% in $^{-}$M plants related to control; at the same

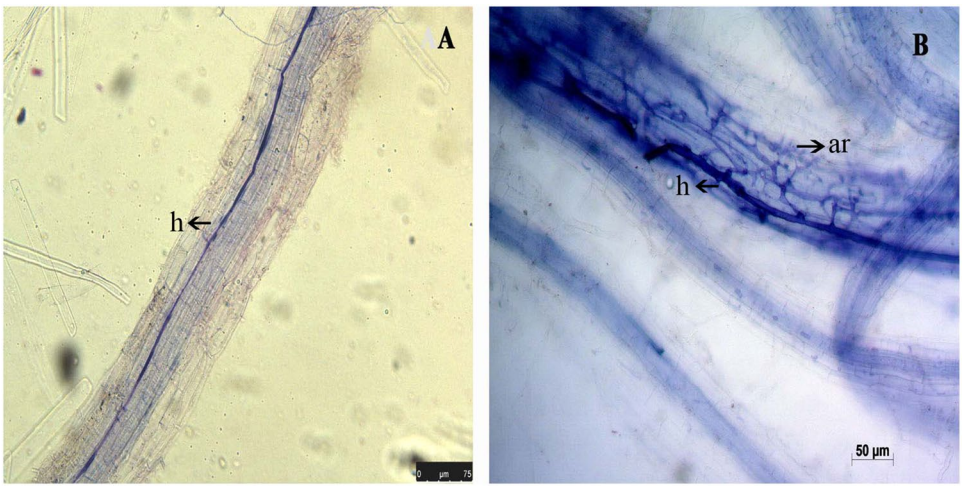


Fig. 1 Micrographs of rice roots associated with *Claroideoglomus claroideum*. **A** Hyphal ramification. **B** Development of arbuscules in roots. h hypahe, ar arbuscules

Table 1 Heavy metal induced changes in the root volume of non AM and AM plants. Values are expressed as mean ± SE of three independent experiments. Different letters following values indicate a significant difference between treatments (Duncan's test, $P \leq 0.05$). The data is an average of recordings from three independent experiments each with ten replicates (i.e., $n = 9$)

	0 day	4 days	8 days	12 days
$^{-}$M plants	2 ± 0.09b	2.1 ± 0.094b	2.2 ± 0.099b	2 ± 0.09c
$^{+}$M plants	4.5 ± 0.18a	4 ± 0.15a	4.2 ± 0.18a	5 ± 0.16a
$^{-}$M Cd plants	2.1 ± 0.094b	1.5 ± 0.067c	1 ± 0.045d	1 ± 0.045d
$^{+}$M Cd plants	4.2 ± 0.189a	3.9 ± 0.155a	2 ± 0.09b	2 ± 0.09c
$^{-}$M Zn plants	2.5 ± 0.125b	1.8 ± 0.081c	1.5 ± 0.067c	1.8 ± 0.081c
$^{+}$M Zn plants	4.5 ± 0.2025a	4.2 ± 0.18a	4 ± 0.18a	3.8 ± 0.20b

time, the root volume of $^{+}$M plants was maintained equal to that of control even under metal stress.

Evans blue uptake was predominantly detected in the roots of Cd and Zn-treated $^{-}$M plants, and it was 141 and 216%, respectively. Moreover, it was only 33 and 171% in the roots of $^{+}$M plants under metal stress (Fig. 2A).

Photosynthesis

Cadmium and Zn stress-elicited reduction of chlorophyll content and photosystems (I and II) activities, and the $^{-}$M plants had a higher reduction compared to $^{+}$M plants. The reduction of total chlorophyll content in $^{-}$M plants and $^{+}$M plants of *O. sativa* was up to 52–60% on 12 days of imparting Cd toxicity. Whereas under Zn toxicity, the reduction was up to 49 and 29% in $^{-}$M plants and $^{+}$M plants, respectively, in comparison with the control (Fig. 2B).

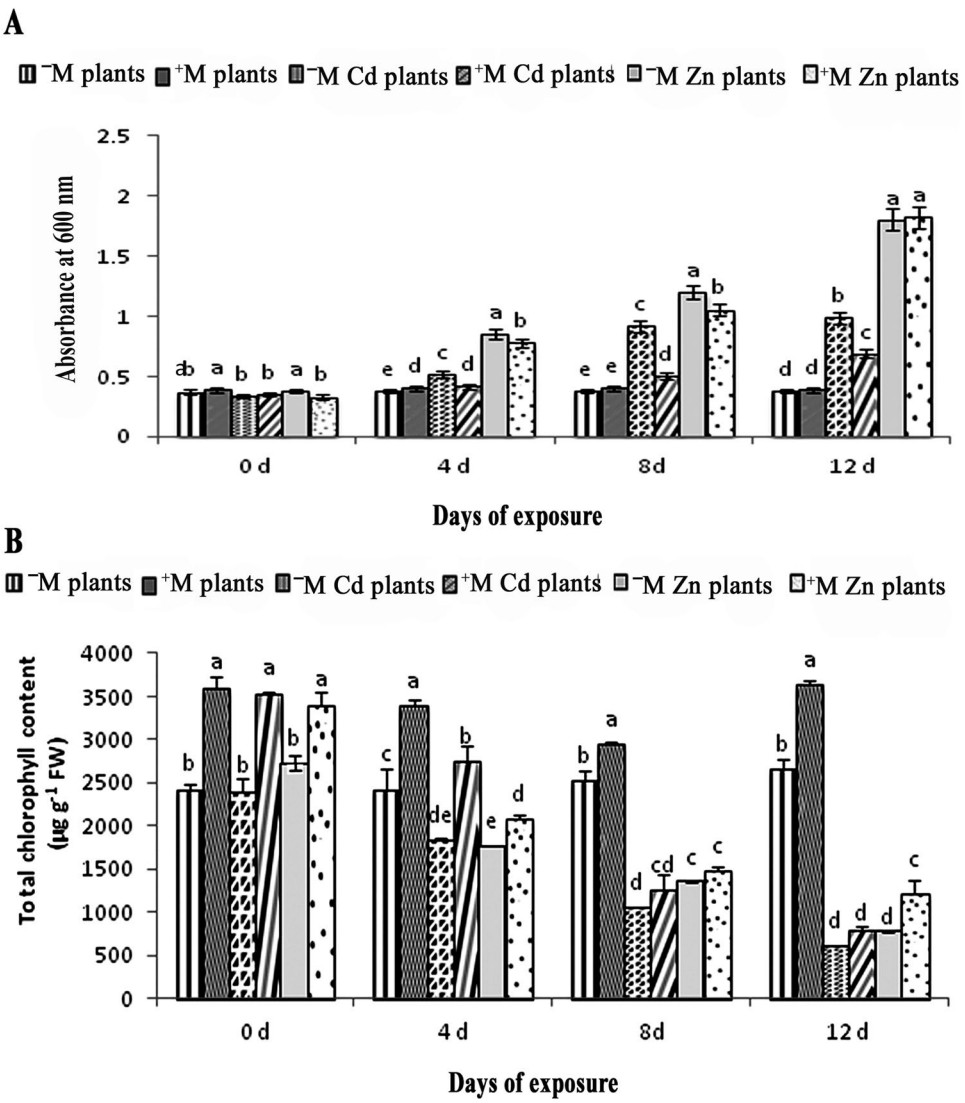


Fig. 2 Rate of cell death and variation in the photosynthetic pigment of rice plants associated with *Claroideoglomus claroideum* exposed to $ZnSO_4$ and $CdCl_2$. **A** Comparison of the root cell death in HM-treated and control plants. **B** Comparison of total chlorophyll in the HM-treated and control plants. Values are expressed as mean ± SE of three independent experiments. Different letters following values indicate a significant difference between treatments (Duncan's test, $P \leq 0.05$)

Detailed interpretations of the photosynthetic response of rice plants treated with heavy metals were carried out using Chl *a* fluorescence analysis (Fig. 3). The Fv/Fm values measured in the leaves of Cd and Zn stresses exposed $^{-}$M plants, and $^{+}$M plants showed only minor reduction related to that of the value recorded in control. The reduction in Fv/Fo was 18–24% in Cd treated $^{-}$M plants as well as in $^{+}$M plants, and it was 52–57% under Zn stress. PI (abs) showed a 25 and 27% reduction in $^{-}$M plants exposed to Cd and Zn stresses, respectively, and the decline was negligible in $^{+}$M plants exposed to Cd. At the same time, the PI (abs) was reduced to 18% in Zn-treated $^{+}$M plants.

Analysis of leaf micromorphological characters revealed that the plants exposed to Cd and Zn stresses had significant variations from the micromorphology of the control plants (Supplementary figures 3, 4, 5, and 6). Partial closure of adaxial stoma was observed in Cd treated plants, whereas it was completely closed in $^{-}$M plants and $^{+}$M plants exposed to Zn stress (Supplementary figure 3). The stomata in the abaxial surface were completely closed in Cd treated $^{-}$M plants (Supplementary figure 4). Any unique structural modification was not observed on the abaxial surface of $^{-}$M plants and $^{+}$M plants under metal stress (Supplementary figure 5). At the same time, the upper leaf surface of $^{-}$M plants developed a unique circular-shaped structure on exposure to Zn, which was not observed in $^{+}$M plants under the same treatment (Supplementary figure 6E).

Superoxides

In the leaves of $^{-}$M plants and $^{+}$M plants, an increase in superoxide content was observed due to the addition of days of exposure to metal toxicity (Fig. 4A). On 8 days of exposure, Cd and Zn treated $^{-}$M plants had a 54 and 72% increase, but the augmentation was only 48 and 67% in $^{+}$M plants. In the root tissues of $^{-}$M plants and $^{+}$M plants, both Cd and Zn induced the accumulation of superoxide content, and it gradually progressed with an increase in the duration of metal stress (Fig. 4B). After 8 days of Cd stress, the augmentation observed in $^{-}$M plants and $^{+}$M plants was 47 and 31%, respectively. However, it was insignificant in $^{-}$M plants and $^{+}$M plants under Zn toxicity. A strong positive correlation was observed between superoxide content and proline in the leaves of $^{-}$M plants ($r = 0.998$, $P \leq 0.01$) and $^{+}$M plants ($r = 0.941$, $P \leq 0.01$).

Hydrogen peroxide

In the leaves of $^{-}$M plants and $^{+}$M plants, Cd as well as Zn stress induced the accumulation of H_2O_2 (Fig. 4C). The increase was 24 and 48% in Cd and Zn treated $^{-}$M plants, whereas, in $^{+}$M plants, the increase was only 11 and 27% on 4 days of metal stress.

Under Cd and Zn toxicity, the roots of $^{-}$M plants and $^{+}$M plants had an increase in H_2O_2 content. The enhancement was 364 and 462% in Cd and Zn exposed $^{-}$M plants, but only 310 and 392% of increase was observed in $^{+}$M plants (Fig. 4D).

MDA

In the leaves of $^{-}$M and $^{+}$M plants, metal stress-induced MDA accumulation (Fig. 4E). The increase in the MDA level was 2- and 4-fold in $^{-}$M plants exposed to Cd and Zn stresses, respectively, whereas only 1- and 3-fold increase was observed in $^{+}$M plants on 8 days of exposure. In contrast, in the roots of $^{-}$M and $^{+}$M plants, a reduction in MDA content was observed on exposure to Cd and Zn stresses. The decrease was 43 and 61% in roots of Cd and Zn treated $^{-}$M plants, whereas the roots of $^{+}$M plants had only a 21 and 48% reduction (Fig. 4F).

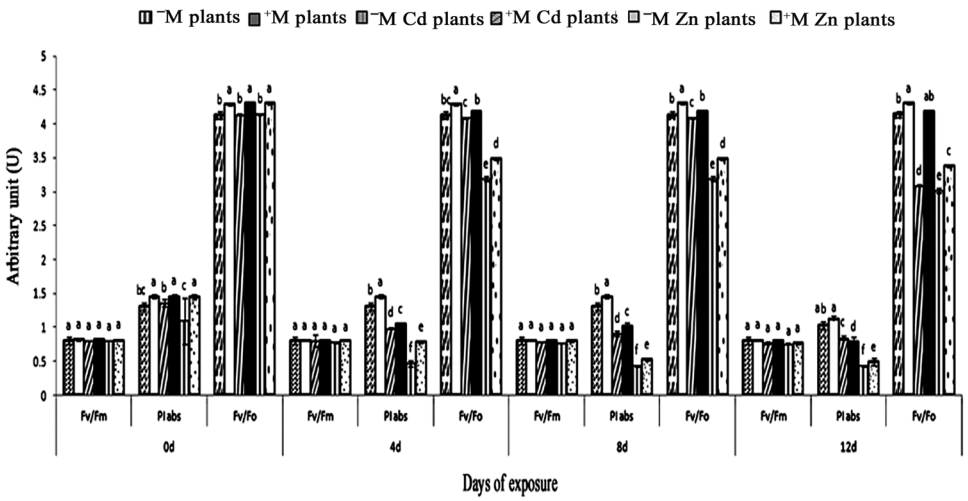


Fig. 3 Different JIP parameters deduced from chlorophyll *a* fluorescence induction curves in rice plants associated with *Claroideoglomus claroideum* exposed to $ZnSO_4$ and $CdCl_2$. Values are expressed as mean ± SE of three independent experiments. Different letters following values indicate a significant difference between treatments (Duncan's test, $P \leq 0.05$)

Primary metabolites

The exposure to the metals elicited alterations in the accumulation pattern of amino acids, proline, and soluble sugar content in the leaves and roots of $^{-}$M and $^{+}$M plants. In the leaves of $^{-}$M and $^{+}$M plants, the soluble sugar content was augmented under Cd and Zn toxicity (Fig. 5A). The increase was 3- and 4-fold in $^{-}$M plants, whereas it was only 2- and 3-fold in $^{+}$M plants on 4 days of metal stress. Whereas, on 12 days of Cd and Zn stresses, more sugar content was observed in $^{+}$M plants as compared to $^{-}$M plants. In the roots of $^{-}$M plants, it was increased on the 4 days of Cd and Zn toxicity, but further, the sugar content had a progressive reduction. Meanwhile, in the roots of $^{+}$M plants, the sugar content was maintained as that of control under Cd and Zn toxicity (Fig. 5B). The MDA content and total soluble sugar had a significant positive correlation in the leaves of $^{+}$M plants ($r = 0.992$, $P \leq 0.01$) and $^{-}$M plants ($r = 0.894$, $P \leq 0.01$) on 8 days of exposure to the metals.

Cadmium, as well as Zn stress, increased the accumulation of amino acids in the leaves of $^{-}$M plants and $^{+}$M plants (Fig. 5C). In $^{-}$M plants, the increase was 9- and 7-fold, whereas it was only 5- and 4-fold in $^{+}$M plants on 4 days of Cd and Zn exposure. Different from the shoot, the amino acid content was decreased (64–65%) in the roots of $^{-}$M plants under Cd and Zn stresses, but the decrease was only 13 and 26% in $^{+}$M plants (Fig. 5D). Total amino acid content of leaves had a strong significant positive correlation with MDA content of $^{-}$M plants ($r = 0.888$, $P \leq 0.05$), but it was insignificant in $^{+}$M plants ($r = 0.645$, $P \geq 0.05$) exposed to metal toxicity.

Both metals induced the accumulation of proline content in the leaves of $^{-}$M and $^{+}$M plants (Fig. 5E). In $^{-}$M plants, the increase in proline content was 9- and 18-fold under Cd and Zn stresses, respectively, in comparison with control. But, there was only 2- and 10-fold increment in the leaves of $^{+}$M plants on 8 days of Cd and Zn treatments. In the roots of $^{-}$M and $^{+}$M plants, the proline content was reduced under metal stress. The reduction was 21–23% in $^{-}$M plants exposed to Cd and Zn, whereas it was only 9–11% in mycorrhizae-associated roots (Fig. 5F).

Secondary metabolites

Both Cd and Zn elicited phenolic accumulation in the leaves, and in $^{-}$M plants, the augmentation in phenolic content was to the extent of 154 and 101%, respectively, whereas in $^{+}$M plants, the increase was only 125 and 76% on exposure to 4 days of Cd and Zn toxicity (Fig. 6A). In the roots of $^{-}$M and $^{+}$M plants, the phenolic content was decreased on exposure to Cd and Zn (Fig. 6B). When compared with the control, the reduction in phenolic content was 25 and 12% in Cd and Zn treated $^{-}$M plants, and there was a negligible reduction in $^{+}$M plants.

The phytochemical composition of leaves and roots of $^{-}$M plants and $^{+}$M plants of *O. sativa* was significantly altered under Cd and Zn stresses (Table 2). Different metabolites found in the leaves of $^{-}$M and $^{+}$M plants include neophytadiene, 3,7,11,15-tetramethyl-2-hexadecen-1-ol, 3,6-octadecadienoic acid, methyl ester, methyl palmitate, phytol, 1,2-benzenedicarboxylic acid di-iso-octyl ester, (E)-phytol, and ethyl iso-allocholate.

Cadmium-induced the accumulation of cholesta-4,6-dien-3-ol, benzoate, (3beta)- and dihydroergosterol in $^{-}$M and $^{+}$M plants. Cadmium stress leads to a drastic reduction in the content of neophytadiene in both $^{-}$M and $^{+}$M plants. Another compound, (E)-phytol, was not detected in $^{-}$M plants, but it was detected only in $^{+}$M plants on exposure to Cd.

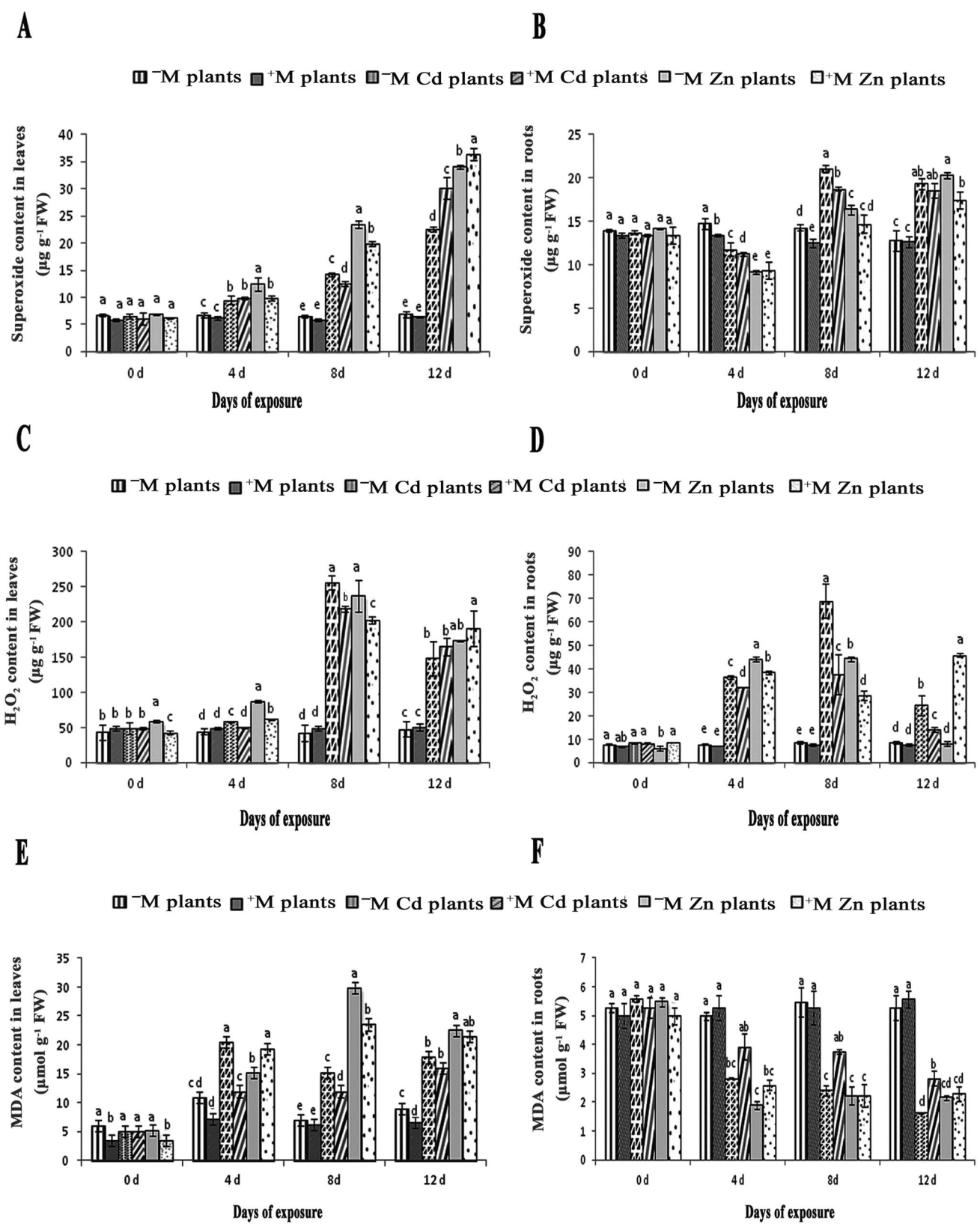
Zinc-induced the accumulation of 3-beta-acetoxystigmasta-4,6,22-triene, 2-pentadecanone, 6,10,14-trimethyl-, and 2-hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]- in the leaves of $^{-}$M and $^{+}$M plants. Whereas, the $^{-}$M plants exposed to Zn stress biosynthesize a unique compound called cyclopropaneoctanoic acid. Moreover, a reduction in neophytadiene content was observed in $^{-}$M plants as well as $^{+}$M plants exposed to Zn toxicity. In $^{-}$M plants exposed to Zn toxicity, the content of phytol was drastically reduced to 3.68%, and it was 21.83% in $^{+}$M plants.

Colonization of mycorrhizae as well as Cd and Zn-induced significant modifications in the metabolic composition of the root (Table 2). Methyl palmitate, 1-heneicosanol, cis-13-octadecenoic acid, methyl ester, methyl stearate, 1,2-benzenedicarboxylic acid, and gamma-sitosterol were commonly observed in $^{-}$M and $^{+}$M plants. Mycorrhization could induce the additional production of 3-beta-acetoxystigmasta-4, 6, 22-triene, and cholesta-4,6-dien-3-ol, benzoate in rice roots.

Under Cd toxicity, stigmasterol was one of the most important chemical constituents observed in non-mycorrhizal roots. Phytol, tributyl acetyl citrate, linoleic acid, and L-norvaline were induced in the mycorrhizal roots. Eicosane, tributyl acetyl citrate, 1,2-benzene dicarboxylic acid, and 3 beta-acetoxystigmasta-4,6,22-triene were the different compounds elicited by elevated Zn toxicity in roots of $^{-}$M and $^{+}$M plants. Besides these, retinol and hexahydrofarnesyl acetone were observed in $^{-}$M plants under Zn toxicity.

Osmolality

Cadmium as well as Zn-induced the accumulation of osmolytes and thus increased the osmolality of leaf cell sap of both $^{-}$M and $^{+}$M plants (Fig. 6C). In $^{-}$M plants, the augmentation in the osmolality was up to 3–3.6-fold, but it was only


A
B
⁻M plants
⁺M plants
⁻M Cd plants
⁺M Cd plants
⁻M Zn plants
⁺M Zn plants
Superoxide content in leaves (µg g⁻¹ FW)
Superoxide content in roots (µg g⁻¹ FW)
0 d
4 d
8d
12 d
Days of exposure
C
D
H2O2 content in leaves (µg g⁻¹ FW)
H2O2 content in roots (µg g⁻¹ FW)
E
F
MDA content in leaves (µmol g⁻¹ FW)
MDA content in roots (µmol g⁻¹ FW)

◂**Fig. 4** Variation in the ROS and MDA content in rice plants associated with *Claroideoglomus claroideum* exposed to $ZnSO_4$ and $CdCl_2$; superoxide content in the leaves (**A**) and roots (**B**) of HM treated and control plants, hydrogen peroxide content in the leaves (**C**) and roots (**D**), and MDA content in the leaves (**C**) and roots (**D**) of heavy metal treated and control plants. Values are expressed as mean ± SE of three independent experiments. Different letters following values indicate a significant difference between treatments (Duncan's test, $P \leq 0.05$)

2–2.5-fold in $^{+}$M plants on 8 days of Cd and Zn exposure. In contrast to leaves, the osmolality in the roots of $^{-}$M plants and $^{+}$M plants was reduced under Cd and Zn toxicity (Fig. 6D). The reduction was 51 and 19% in Cd and Zn treated $^{-}$M plants, but in $^{+}$M plants, a negligible reduction was recorded. The correlation between osmolality and MDA was positive in the shoots of $^{-}$M plants ($r = 0.706$, $P \leq 0.05$) and $^{+}$M plants ($r = 0.710$, $P \leq 0.05$) on 8 days of metal stress. But in the case of roots, the correlation between osmolality and MDA was negative in $^{-}$M plants ($r = -0.705$, $P \leq 0.05$) and positive in $^{+}$M plants ($r = 0.775$, $P \leq 0.05$).

Estimation of Cd and Zn in plant tissue

In rice plants, the accumulation of Cd and Zn gradually increased according to the increase in the duration of Cd and Zn stresses (Table 3). In the roots of $^{-}$M plants, Cd ion accumulation was 3.13 ± 0.16 mg g^{-1} DW, and it was 2.25 ± 0.11 mg g^{-1} DW in $^{+}$M plants. As compared to roots, leaves of rice plant accumulated low concentrations of Cd in the shoot. The cadmium concentration found in the leaves of $^{-}$M plants was 0.9657 ± 0.03 mg g^{-1} DW, and it was 0.8210 ± 0.05 mg g^{-1} DW in $^{+}$M plants on 12 days of exposure (Table 3).

In the roots of $^{-}$M plants, the rate of Zn accumulation was 1.5591 ± 0.08 mg g^{-1} DW, and maximum Zn accumulation was observed in $^{+}$M plants (1.8604 ± 0.09 mg g^{-1} DW). The accumulation of Zn in the leaves was minimum in $^{-}$M (0.2748 ± 0.03 mg g^{-1} DW) and $^{+}$M plants (0.2309 ± 0.02 mg g^{-1} DW). In a normal growth environment, the translocation factor (TF) of Zn ions was higher in $^{+}$M plants (0.96) as compared to $^{-}$M plants (0.81). Under Zn toxicity, the TF of Zn ions was decreased in $^{-}$M plants, and $^{+}$M plants and it was 0.18 and 0.12 in $^{-}$M plants and $^{+}$M plants, respectively. However, the TF of Cd was 0.31and 0.36 in $^{-}$M plants and $^{+}$M plants, respectively, on 12 days of Cd stress.

Discussion

Effects of AM on growth and photosynthesis of rice exposed to Cd and Zn stressors

In the current work, 1.95 g Zn kg^{-1} soil and 0.45 g Cd kg^{-1} soil were screened as stress imparting concentrations from various Cd and Zn concentrations, but the stress imparting concentrations of metal in various other studies were different from ours. For example, 1050 µg mL^{-1} Cd was selected as the minimal inhibitory concentration of Cd by Mitra et al. (2018), and it was 15 µg Cd^{2+} g^{-1} dwt by Dell'Amico et al. (2008). These deviations in the stress imparting concentration depend on the duration of the study, soil type, plant species, etc. The shoot fresh weight of both $^{+}$M plants and $^{-}$M plants was lower than that of control under Cd and Zn stress. Under heavy metal stresses, the fresh weight and dry weight of the plants are reduced due to the reduction in plant growth and cell division (Latef 2013). The reduction in the shoot fresh weight could be attributed to the blockage in the xylem vessels, which hinders the transport of water and minerals toward the shoot (Subramanian et al. 2009). The root fresh weight reduction due to Cd and Zn toxicity was also attributed to the reduced cell division. The increase in water content of the roots was directly related to the "root dilution mechanism" wherein more water is taken up into the cells in order to dilute the high metal ion concentration in tissues. Moreover, the increased water content reduces the osmotic potential of roots by reducing the solute potential of root cytoplasm (Filipović 2020). This root dilution mechanism was more predominant in $^{-}$M plants than $^{+}$M plants so as to withstand the elevated Cd and Zn concentrations. In the current study, mycorrhization reduced the decrease in fresh weight and moisture content of the leaves due to the Cd and Zn stresses, meaning that the water transport to the shoots was not hindered as that of $^{-}$M plants. These findings were supported by the results obtained from the experiment conducted in *Trigonella foenum-graecum* (Abdelhameed and Metwally 2019). Under Cd stress, the fresh weight of shoot and root was reduced in *T. foenum-graecum*, but mycorrhization significantly decreased the reduction in plant fresh weight.

Cadmium, as well as Zn toxicity, reduced the photosynthetic efficiency of both $^{-}$M plants and $^{+}$M plants by reducing chlorophyll and carotenoid content, electron transport efficiency, and stomatal opening. Cd toxicity prevents the synthesis of chlorophyll by the replacement of Mg^{2+} with Cd ions (Grajek et al. 2020). Moreover, Cd toxicity leads to a decrease in the biosynthesis of chlorophyll, and it also inhibits the activity of some enzymes of the Calvin cycle (Baryla et al. 2001). Zinc toxicity caused a decline in chlorophyll content of $^{-}$M plants and $^{+}$M plants, and similar toxic effects of Zn on chlorophyll levels were reported in *Miscanthus* × *giganteus* plants (Andrejić et al. 2018). In $^{+}$M plants, the reduction of chlorophyll was less as compared to $^{-}$M plants under heavy metal stress. The mycorrhizae-mediated stabilization of metals in roots or rhizosphere helps the $^{+}$M plants to avert the excess presence of metal ions in leaves, which can inhibit chlorophyll biosynthesis. AM aided in the maintenance of chlorophyll content without any significant

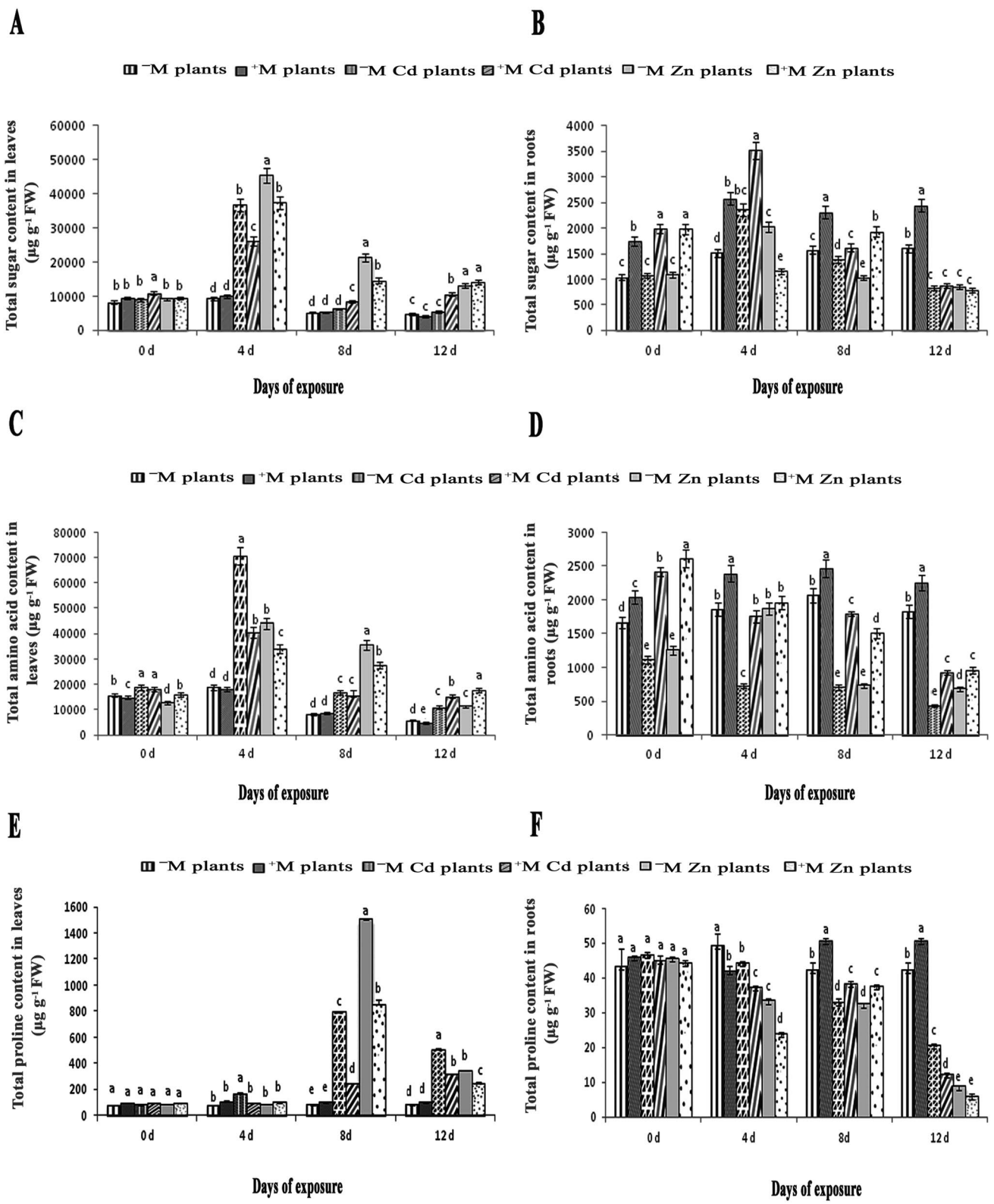


Fig. 5 Metabolic alterations observed in rice plants associated with *Claroideoglomus claroideum* exposed to $ZnSO_4$ and $CdCl_2$; total soluble sugar content in the leaves (**A**) and roots (**B**) of HM treated and control plants, total free amino acid content in the leaves (**C**) and roots (**D**) of HM treated and control plants and total proline content in the leaves (**E**) and roots (**F**) of heavy metal treated and control plants. Values are expressed as mean ± SE of three independent experiments. Different letters following values indicate a significant difference between treatments (Duncan's test, $P \leq 0.05$)

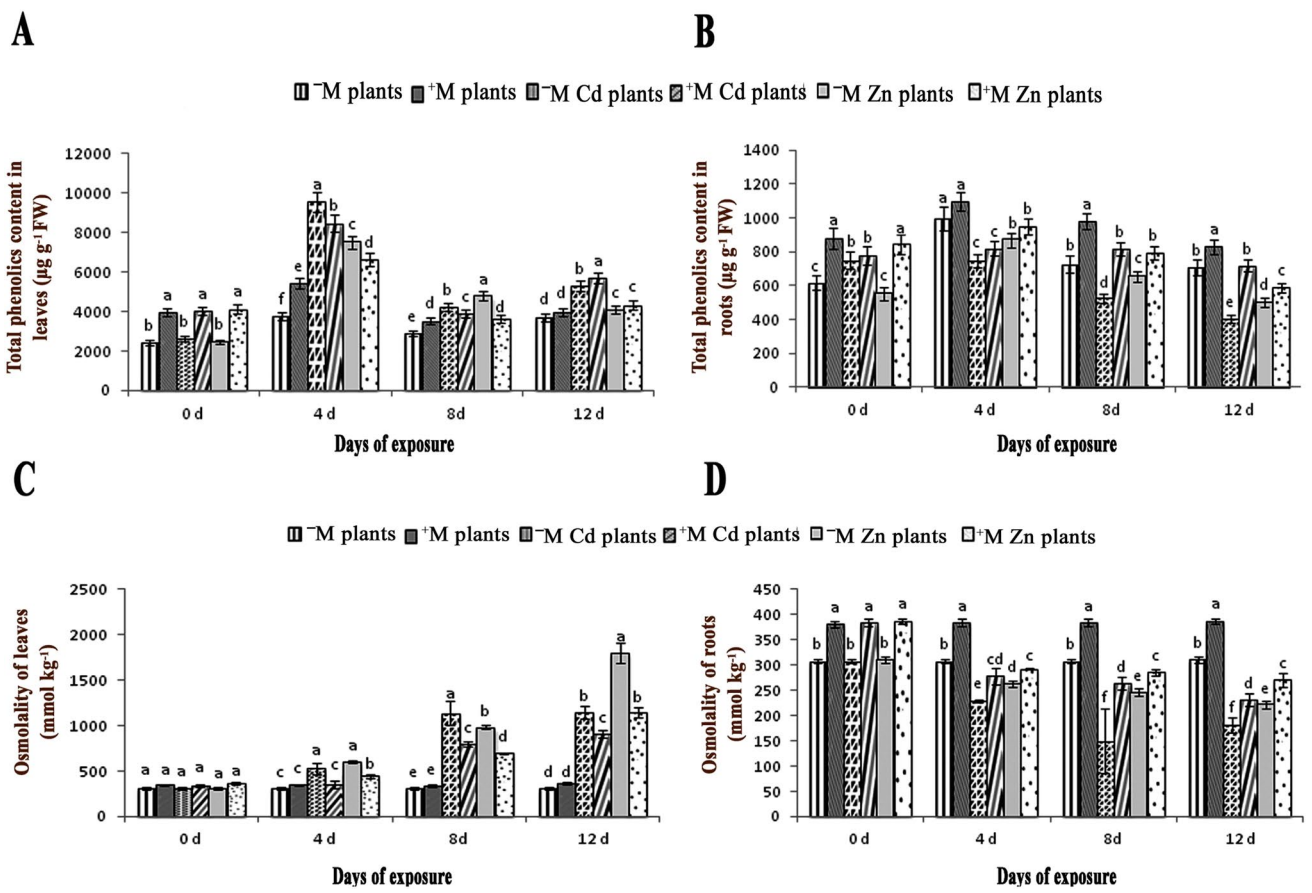


Fig. 6 The phenolics content and osmolality observed in rice plants associated with *Claroideoglomus claroideum* exposed to $ZnSO_4$ and $CdCl_2$; phenolics content in the leaves (**A**) and roots (**B**) of HM treated and control plants, and osmolality in the leaves (**C**) and roots (**D**) of HM treated and control plants. Values are expressed as mean ± SE of three independent experiments. Different letters following values indicate a significant difference between treatments (Duncan's test, $P \leq 0.05$)

reduction in *Cajanus cajan* under Cd and Zn toxicity (Garg and Singh 2018).

Stomatal closure was the most significant limitation to photosynthesis under Cd and Zn toxicity (Sagardoy et al. 2010). In the current study, both Cd and Zn induced stomatal closure in $^{-}$M plants and $^{+}$M plants, but $^{+}$M plants maintained partial stomatal opening under Cd and Zn toxicity and thus retained the photosynthetic efficiency. The hydro-active closure of stomata is a direct effect of water loss and desiccation of plants (Stålfelt 1955; Buckley 2005). The closure of stoma can be correlated with the tissue moisture content, which was drastically reduced in $^{-}$M plants related to the $^{+}$M plants. Heavy metal-induced stomatal closure was associated with a restriction in the water uptake due to the damaged root cells (Barceló and Poschenrieder 1990). Mycorrhizae induced stomatal opening in the host plant under abiotic stresses, which was reported in previous works, and this is aided by the symbiont by facilitating water uptake under extreme stress conditions (Dhalaria et al. 2020). Mycorrhizae induced soil aggregate formation and the extensive ramification of hyphae improve the water holding capacity of soil and increase surface area for water absorption, which aid the plants to tolerate heavy metal stress (Leyval et al. 1997). Analysis of leaf micromorphological characters revealed the presence of circular-shaped special structures in Zn-treated $^{-}$M plants, which further reduced the photosynthetic efficiency. The structural modification in the leaf micromorphology was a typical response of plants towards heavy metal stress (Rai and Mehrotra 2008; Sruthi and Puthur 2019). Ultramorphological changes such as reduction in the wax deposition and widening of stomatal opening were observed under Cr toxicity in *Phyllanthus amarus* (Rai and Mehrotra 2008). At the same time, Pb induced an increase in the number of stomata in soybean leaves (Weryszko-Chmielewska and Chwil 2005). The specific leaf modification observed in the present study is being reported for the first time in rice under Zn toxicity. Interestingly, it was not observed in rice plants exposed to Cd toxicity. The zinc-induced accumulation of volatile

Table 2 Metabolic modifications in rice plants associated with mycorrhiza exposed to $ZnSO_4$ and $CdCl_2$

Bioactive compounds	Area percentage (%)					
	$^{-}$M plants	$^{+}$M plants	$^{-}$M Cd plants	$^{+}$M Cd plants	$^{-}$M Zn plants	$^{+}$M Zn plants
Neophytadiene	44.5	40.88	18.27	20.68	30.81	31.32
(22e)-Stigmasta-4,6,22-trien-3-yl acetate	-	-	-	-	1.44	-
(E)-phytol	10.9	11.15	-	7.85	13.95	10.26
1,2-Benzenedicarboxylic acid, diisooctyl ester	3.47	5.38	2.75	-	-	-
22-Dihydroergosterol	-	-	-	3.06	-	-
2-Hexadecen-1-ol, 3,7,11,15-tetramethyl	-	-	7.74	-	-	16.11
2-Pentadecanon, 6,10,14-trimethyl-	-	-	-	-	4.14	4.42
3,6-Octadecadienoic acid, methyl ester	3.2	-	-	-	0	0
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	7.28	7.46	5.43	9.28	8.84	16.11
3.Beta.-acetoxystigmasta-4,6,22-triene	-	-	-	-	-	2.93
9,12-Octadecadienoic acid (z,z)-, methyl ester	-	-	-	-	1.59	-
Cholesta-4,6-dien-3-ol, benzoate, (3.beta.)-	-	-	2.55	2.09	-	2.15
Cyclopropaneoctanoic acid, 2-[[2-[(2-ethylcyclopropyl) methyl]cyclopropyl]methyl]-, methyl ester	-	-	-	-	2.73	-
Dihydroergosterol			3.22	-	-	-
Ethyl iso-allocholate	1.73	1.37	-	-	-	-
Hexadecadienoic acid, methyl ester	-	2.75	-	-	3.73	-
Hexadecanoic acid	-	-	5.81	-	-	-
Linoleic acid, methyl ester	-	-	-	-	-	1.41
Methyl isoheptadecanoate	-	3.33	-	-	-	-
Methyl linolenate	-	-	-	-	-	3.17
Methyl palmitate	3.75	-	-	-	-	5.38
Methyl stearate	-	-	-	-	-	0.88
Phytol	25.17	27.69	52.38	56.13	3.68	21.83
Squalene			1.85	0.91	1.74	-
Methyl palmitate	38.42	29.52	15.48	26.39	-	17.73
Gamma-sitosterol	29.14	-	7.93	-	6.14	-
1,2-Benzenedicarboxylic acid	9.66	14.74	7.26	11.98	21.81	25.98
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	-	6.78	-	-	7.57	-
10-Nonadecanone	-	-	-	-	-	3.98
Octadecenoic acid, methyl ester	6.87	12.42	3.63	8.39	8.94	10.13
1-Heneicosanol	3.53	-	-	3.03	1.06	7.49
2-Methyloctacosane	-	-	-	1.75	-	-
2-Pentadecanone, 6,10,14-trimethyl-	-	1.82	-	-	-	-
3.Beta-acetoysgmasta-4,6,22-triene	-	2.57	-	2.57	-	2.62
4,22-Stigmastadiene-3-on	-	-	2.26	-	-	-
8-Octadecanone	-	-	-	-	-	4.92
9,12-Octadecadienoic acid, methyl ester	-	5.1	-	-	5.35	-
9-Eicosene, (e)-	-	-	-	-	1.79	-
Cholesta-4,6-dien-3-ol, benzoate, (3.beta.)-	-	1.54	-	1.87	2.03	-
Cyclopentanone, 3-(2-oxopropyl)-	-	-	5.02	-	-	-
E-15-Heptadecenal	-	-	-	-	-	7.66
Eicosane	-	4.29	2.12	-	1.68	2.41
Eicosane, 2-methyl-	-	-	-	2.5	-	-
Ethyl iso-allocholate	-	-	4.51	-	-	-
Gamma-sitosterol	29.14	-	7.93	-	6.14	-
Heptadecane	-	3.78	-	-	-	-
Hexadecanoic acid, methyl ester	-	-	-	-	16.5	-

Table 2 (continued)

Bioactive compounds	Area percentage (%)					
	$^{-}$M plants	$^{+}$M plants	$^{-}$M Cd plants	$^{+}$M Cd plants	$^{-}$M Zn plants	$^{+}$M Zn plants
Hexahydrofarnesyl acetone	-	-	-	-	4.05	-
Linoleic acid, methyl ester	-	-	-	4.2	-	-
L-Norvaline, N-(2-methoxyethoxycarbonyl)-, hexadecyl ester	-	-	-	2.89	-	-
Methyl 18-methylnonadecanoate	-	-	-	2.14	-	-
Methyl lignocerate		2.75	5.26	2.95	2.31	-
Methyl stearate	12.34	13.08	8.04	15.46	6.16	5.81
Neophytadiene	-	1.54	-	-	-	-
N-hentriacontanol-1	-	-	1.87	-	-	-
Nonadecane	-	-	-	2.33	-	-
Octadecane	-	-	-	-	-	2.192
Oleic acid, propyl ester	-	-	9.79	-	-	-
Pentadecane	-	-	-	-	1.7	-
Phytol	-	-	-	5.94	2.09	5.23
Phytol, acetate	-	-	-	1.34	1.17	-
Retinol, acetate	-	-	-	-	2.18	-
Solanesol	-	-	-	-	2.57	-
Stigmast-5-en-3-ol, (3.beta.,24s)-	-	-	12.31	-	-	-
Stigmasta-5,22-dien-3-ol	-	-	14.46	-	-	-
Tetracosane, 3-ethyl-	-	-	-	2.54	-	-
Tributyl acetylcitrate	-	-	-	1.7	1.73	1.91

compounds could have led to the development of this structure, through which the emission of these volatile organic compounds may be possible. Zinc potentially induced the volatile compound accumulation in *Martianthus leucocephalus* as compared to Cd (Jesus et al. 2016). An increase in the methanol, propanal, acetylene, and acetaldehyde content in the leaves of *Tetradenia riparia* on exposure to Zn stress also supports our hypothesis (Bibbiani et al. 2018). The absence of these structures in $^{+}$M plants indicates less toxicity due to a lower Zn concentration in the leaf cytoplasm as compared to the $^{-}$M plants.

Chlorophyll *a* fluorescence parameters like Fv/Fm, Fv/Fo, and PI_{ABS} are reduced in Cd- and Zn-exposed $^{-}$M plants and $^{+}$M plants. The reduction in the Fv/Fm and PI_{ABS} indicates the reduction in the efficiency of PSII, and the reduction was greater in $^{-}$M plants in comparison with $^{+}$M plants. Cd and Zn induced reduction in the activity of the PSII donor side was reported by Pauno et al. (2018). In the current study, Cd and Zn significantly inhibited the PSII donor side, but the decrease was very prominent in $^{-}$M plants as compared to $^{+}$M plants. A parallel result was observed in *Triticum aestivum* associated with *Glomus mosseae*, where heavy metal-induced reduction in Fv/Fm and PI_{ABS} was overcome with the help of mycorrhization (Shahabivand et al. 2012a, b). $^{+}$M plants potentially reduced ROS development and thus reduced the degradation of thylakoid membranes, maintaining the integrity of PSI and PSII (Shahabivand et al. 2012a, b).

Oxidative stress and antioxidation mechanisms

The increase in MDA content in rice leaves signifies oxidative stress. MDA is an indicator of lipid peroxidation or membrane degradation by ROS, and the accumulation of ROS can result due to heavy metal toxicity (Lombardi and Sebastiani 2005; Tripathi et al. 2020; Janeeshma et al. 2022). When peroxidation of lipid occurs via free radicals, numerous reactions are initiated, which cause damage to fatty acids and finally damage to biological membranes (Shewfelt and Purvis 1995). In this study, an increase in MDA content was observed in AM and $^{-}$M plants subjected to Cd and Zn treatment, but $^{-}$M plants exhibited a higher accumulation of MDA than $^{+}$M plants, which indicates increased oxidative stress in the former. Moreover, Zn induced a prominent augmentation in the ROS accumulation and MDA content compared to Cd-treated plants. The enhancement in the level of superoxide and H_2O_2 content showed a direct correlation with the enhancement of MDA content. Mycorrhization decreased the accumulation of superoxide and H_2O_2 content in leaves of rice, and accordingly, MDA accumulation was also reduced in $^{+}$M plants. MDA content was decreased in the roots under heavy metal exposure, and this

Table 3 Bioaccumulation of Cd and Zn (mg g^{-1} DW) in non-AM and AM *O. sativa* associated with mycorrhiza exposed to $CdCl_2$ (0.45 g kg^{-1}) and $ZnSO_4$ (1.95 g kg^{-1}) toxicity. BDL indicates below detectable level. Values are expressed as mean ± SE of three independent experiments. Different letters following values indicate a significant difference between treatments (Duncan's test, $P \leq 0.05$). The data is an average of recordings from three independent experiments each with ten replicates (i.e., $n = 9$)

		0 day			4 days			8 days			12 days		
		Root	Shoot	TF	Root	Shoot	TF	Root	Shoot	TF	Root	Shoot	TF
$^{-}$M plants	Zn	0.0827 ± 0.008b	0.0708 ± 0.01b	0.86	0.0827 ± 0.005d	0.0664 ± 0.01d	0.80	0.0807 ± 0.01c	0.0624 ± 0.01d	0.77 b	0.0817 ± 0.002d	0.0665 ± 0.01d	0.81
$^{+}$M plants		0.0997 ± 0.004a	0.0885 ± 0.01a	0.89	0.1028 ± 0.01c	0.0885 ± 0.01c	0.86	0.0993 ± 0.01c	0.0885 ± 0.01c	0.891	0.0922 ± 0.002c	0.0885 ± 0.01c	0.96
$^{-}$M Zn plants		0.0827 ± 0.008 b	0.0664 ± 0.01c	0.80	0.5937 ± 0.03a	0.2148 ± 0.02a	0.36	0.9184 ± 0.05b	0.3659 ± 0.04a	0.40	1.5591 ± 0.08b	0.2748 ± 0.03a	0.18
$^{+}$M Zn plants		0.0997 ± 0.02a	0.0885 ± 0.01a	0.88	0.3275 ± 0.02b	0.2013 ± 0.02a	0.61	1.248 ± 0.06a	0.2849 ± 0.03b	0.23	1.8604 ± 0.09a	0.2309 ± 0.02b	0.12
$^{-}$M plants	Cd	BDL	BDL		BDL	BDL		BDL	BDL		BDL	BDL	
$^{+}$M plants		BDL	BDL		BDL	BDL		BDL	BDL		BDL	BDL	
$^{-}$M Cd plants		BDL	BDL		0.3614 ± 0.02a	0.6291 ± 0.031a	1.74	4.293 ± 0.21a	0. 748 ± 0.05a	0.17	3.127 ± 0.16a	0. 966 ± 0.03a	0.31
$^{+}$M Cd plants		BDL	BDL		0.3772 ± 0.02a	0.4677 ± 0.023b	1.24	3.221 ± 0.16b	0. 694 ± 0.048b	0.22	2.255± 0.11b	0.821± 0.05b	0.36

could be correlated to the reduced live root cells, which was evidenced in the Evans blue staining of the root tissue. Similar results of reduced viable cells were observed in the rice root tissues exposed to Cu stress (Chen et al. 2004a, b). However, the ROS content increased in the root tissues, indicating the enhancement of the oxidative stress imparted by Cd and Zn toxicity, and mycorrhization helps to reduce ROS generation and thus protect the root cells from oxidative accumulation stress. Parallel to our results, mycorrhizae-induced decrease in ROS accumulation was reported in *Solanum lycopersicum* and *Cajanus cajan* exposed to heavy metal stress (Garg and Bhandari 2012; Kumar et al. 2015b). The mycorrhizae-induced reduction in ROS content was due to the increase in the chelation of toxic metal ions, which reduces their bioavailability to plants; simultaneously, this symbiotic association potentially elicits the production of different antioxidants in roots, which also helps to maintain the ROS level in the cytoplasm (Chen et al. 2004a, b).

Osmotic adjustment

Heavy metal stress-elicited accumulation is widely observed in different plants (Girija et al. 2002; Roychoudhury and Tripathi 2020). The disturbance in the water balance of leaves due to the deposition of excess Cd and Zn in the xylem induces proline biosynthesis. Proline accumulation in plant tissue occurs as a result of augmentation in proline biosynthesis and also by protein hydrolysis (Charest and Ton Phan 1990). In the present study, a sharp increase in the proline content was observed under Zn stress, whereas Cd induced a gradual increase. This special influence of Zn ion in proline biosynthesis was observed in wheat plants exposed to 5 mg L^{-1} of Cd and Zn (Zhao et al. 2011), and this result supports our findings. At the initial stages of metal stress, $^{-}$M plants absorbed a high level of Cd and Zn ions and translocated them to the shoot, and to encounter the same, more proline was synthesized. Similar results were found in *Cassia italica* under Cd stress (Hashem et al. 2016a). At the latter stages of metal exposure, proline accumulation was at a higher level in $^{+}$M plants. This result indicates an uninterrupted proline biosynthesis in $^{+}$M plants and a metal-induced decrease in the proline biosynthesis of $^{-}$M plants.

In the roots, the proline content is dramatically reduced, especially in $^{-}$M plants treated with Cd and Zn stresses. Similarly, when rice was exposed to copper stress, a reduction in proline content of roots was observed, and external application of proline helps to protect roots from damage by Cu toxicity (Chen et al. 2004a, b). But, in the present study, the association with mycorrhiza lowered the decrease in the proline content under Cd and Zn toxicity, which significantly contributes to the metal tolerance capacity of rice plants. In *Cajanus cajan, Helianthus annuus,* and *Solanum lycopersicum* proline synthesis was

induced as a result of association with mycorrhizae (Ef et al. 2015; Hashem et al. 2016a; Garg and Singh 2018). Mycorrhizae-induced proline accumulation could be attributed to the inhibition of proline dehydrogenase and an increase in the activity of proline biosynthetic enzymes (delta 1-pyrroline-5-carboxylate synthetase and glutamate dehydrogenase) (Garg and Baher 2013).

Plants exposed to heavy metal accumulation contain a high amount of N-metabolites, which include amino acids, peptides, and amines, and these molecules have three major functions, namely metal chelation, antioxidant defense, and signalling (Sharma and Dietz 2006). The major amino acids produced in plants during heavy metal stress are proline and histidine. Ni-hyperaccumulator *Allium* sp. showed increased accumulation of histidine during heavy metal toxicity (Kramer et al. 2003). In the current study, amino acids drastically accumulated in $^{-}$M plants, but the increase in $^{+}$M plants was low. Similar results of increased proline and total amino acid content were observed in jack bean plants on exposure to elevated concentrations of Zn in the $^{-}$M plants as compared to $^{+}$M plants (Andrade et al. 2009). These observations point out the fact that $^{+}$M plants could counter the heavy metals in a way totally different from that of $^{-}$M plants. The stabilization of heavy metals in the hyphae and other fungal structures in $^{+}$M plants could reduce the toxicity, which does not warrant the enhanced synthesis of amino acids. Moreover, the AM-induced reduction in metal transport towards the shoot was evident in this study, and hence there is no role for overaccumulated amino acids in $^{+}$M plants.

Cadmium-induced enhancement of soluble sugar content in *Cajanus cajan* was reported by Garg and Chandel (2012), and they recorded higher sugar content in $^{+}$M plants as compared to $^{-}$M plants. In the current study, the soluble sugar content was higher in the leaves of $^{-}$M plants in the early stages of Cd and Zn stress exposure. The soluble sugar accumulation was triggered in response to the ROS outbreak, and it stabilizes cellular membranes (Keunen et al. 2013). Therefore, the elevated ROS accumulation could be a major reason for the increased biosynthesis of sugar content in $^{-}$M plants. A similar result in the sugar content was observed in *Pistacia vera.* On exposure to Cd, the total sugar compounds were increased in the shoot, and this sugar accumulation was less in mycorrhizal plants (Rohani et al. 2019). The accumulation of sugars, even when there was a reduction in photosynthesis under Cd and Zn stresses, could be largely contributed by the excessive degradation of starch (Dong and Beckles 2019). The root sugar content of $^{-}$M plants and $^{+}$M plants was reduced in roots on exposure to heavy metals, which was related to the probable reduction in translocation of sugars from the shoot due to the reduction in photosynthesis. Moreover, sugar formed by the starch degradation would be utilized by the shoot itself, and there was minimum to be transported to the roots.

The reduction of proline, amino acids, and sugars in $^{+}$M plants as compared to $^{-}$M plants does not indicate a reduced tolerance of $^{+}$M plants towards heavy metal stress. It has to be understood that $^{+}$M plants are not encountering the same stress situation as that of $^{-}$M plants, which is very clear from the lower MDA content recorded in the former as compared to the latter. When the stress impacts are less in mycorrhiza-associated plants, the need for the overproduction of various metabolites to counter the stress is also less synthesized, as reflected in this study. Similar to our results, AM-associated *T. aestivum* showed less metabolite content than $^{-}$M plants on exposure to Cd stress (Kanwal et al. 2015). The variation in the rate of metabolite accumulation between Cd and Zn treated plants indicates the differential tolerance responses of rice plants towards Cd and Zn treatments. In the case of roots, the reduction of osmolality and osmolyte accumulation has a strong relation with the enhancement of tissue damage by the oxidative stress induced by Cd and Zn ions. Comparably the case of $^{+}$M plants, the intensity of oxidative stress was less, as indicated by the reduced accumulation of ROS.

Secondary metabolites in response to metal toxicity

Rice elicited the biosynthesis of leaf phenolic compounds under Cd and Zn stresses (Chen et al. 2019a). Plant phenolics can directly scavenge the ROS overaccumulated in plant tissues under Cd and Zn stresses (Hiba et al. 2021). Different phenolic compounds are considered strong metal chelators, which boost the Cd and Zn stress tolerance of plants (Kısa et al. 2016). In the early stage of metal exposure, total phenolic content was augmented in $^{-}$M plants, coinciding with higher ROS production. And in $^{+}$M plants, the ROS and phenolics accumulated were less under Cd and Zn stresses. A coinciding result in the phenolics content was observed in *Pistacia vera.* On exposure to Cd stress, phenolic content in the shoot was drastically increased in *Pistacia vera,* but it was less in mycorrhizal plants (Rohani et al. 2019). Similar findings were observed in *Cassia italica* exposed to elevated concentrations of Cd (Hashem et al. 2016b). But, on 12 days of Cd and Zn exposure, $^{+}$M plants had higher phenolic content as compared to $^{-}$M plants. indicating uninterrupted biosynthesis of phenolic operational in $^{+}$M plants but not in $^{-}$M plants.

In the case of both mycorrhizal and non-mycorrhizal roots, the phenolic content was decreased. Similarly, elevated concentrations of boron and aluminum decreased the phenolic content of *Linum usitatissimum* roots by reducing the activity of enzymes involved in the phenyl proponoid pathway (Heidarabadi et al. 2011). The elevated levels of Cd and Zn above the tolerance limit would have reduced

the biosynthesis process of phenol compounds in the roots of $^{-}$M plants.

Secondary metabolite profiling of Cd- and Zn-treated shoots was carried out to study the differential response of $^{-}$M plants and $^{+}$M plants towards these metals. Neophytadiene is a sesquiterpene with three consecutive isoprene units. The reduction of neophytadiene with the increase of dihydroergosterol under Cd treatment indicates the degradation of neophytadiene aids in the biosynthesis of dihydroergosterol. Dihydroergosterol maintains the permeability and fluidity of the plasma membrane by conjugating with the phospholipids, which improves the stress tolerance of plants (Bartram et al. 2006; Aboobucker and Suza 2019). Phytol is the side chain of chlorophyll, and it is a product of pigment hydrolysis metabolism. Phytol, with two different retention times (Rt), was identified in the leaf extract of $^{-}$M plants and $^{+}$M plants, (E)-Phytol (Rt-27.387) and phytol (Rt-31.849). Lippold et al. reported that the modification of phytol into fatty acid phytol esters is elicited by different abiotic stresses (2012) and this could be the reason for the reduction in the content of phytol. The higher Rt value of the second form of phytol represents the stress-induced addition of lengthy hydrocarbon chains in the fatty acids of the thylakoid membrane (Lippold et al. 2012), and only this form of phytol was detected in Cd treated $^{-}$M plants. These findings indicate the accumulation of fatty acid phytol esters as a result of stress induced by Cd, whereas in Cd treated $^{+}$M plants, both forms were detected.

In Zn treated $^{-}$M plants, the concentration of phytols was reduced, which indicates the higher vulnerability of these plants towards Zn toxicity over $^{+}$M plants. Zn induced the accumulation of 30beta-acetoxystigmasta-4,6,22-triene, which has the potential to elicit the plasma membrane H^{+}-ATPase, aiding proton transportation (Aboobucker and Suza 2019). Cyclopropaneoctanoic acid is a representative of cyclic fatty acid, determining the physicochemical characters of membrane and was characteristic of Zn treated $^{-}$M plants, which also supports the oxidative stress developed in $^{-}$M plants. The metal-induced elicitation of various bio compounds described above was conspicuous in the shoots of $^{-}$M plants, and it could be due to the higher concentration of Cd and Zn ions translocated to the shoot system, imparting more severe stress conditions.

As the root is directly interacting with the mycorrhizae and heavy metals, it shows drastic changes in the quality of secondary metabolites. Stigmasterol is considered a strong signal for cellular defense (Aboobucker and Suza 2019), and in this study, it was detected in $^{-}$M plants under Cd stress. Drought-induced increase in the stigmasterol content was reported in rice, and it helps to increase stress tolerance (Kumar et al. 2015a). The presence of tributyl acetyl citrate in mycorrhizal plants under Cd stress indicates the overproduction and exudation of organic acids, which help in the chelation of metal ions (Ma et al. 1997; Javed et al. 2017). The role of jasmonic acid in stress tolerance was explained by many researchers, and its precursor linoleic acid was augmented in mycorrhizal plants treated with Cd stress (Kontos and Spyropoulos 1996). Both $^{-}$M plants and $^{+}$M plants accumulated tributyl acetyl citrate, 1,2-benzene dicarboxylic acid, and 3-beta-acetoxystigmasta-4,6,22-triene, compounds with antioxidant properties that help to tolerate the elevated level of Zn ions in the soil. Retinol showed a significant increase in *Phaseolus vulgaris* on exposure to different heavy metals (Zengin and Munzuroglu 2005), and in the present study, a notable increase of this secondary metabolite was seen in the roots of non-mycorrhizal plants under Zn stress. Hexahydrofarnesyl acetone, an aliphatic ketone, has a potential role in the antioxidation machinery and it is increased in non-mycorrhizal plants under Zn stress (Singh et al. 2009). The above results showed that non-mycorrhizal and mycorrhizal plants responded differentially toward Cd and Zn stresses, concerning the accumulation of various secondary metabolites having varied roles in taking care of metal toxicity.

A decrease in the Cd accumulation by the root with the colonization of mycorrhiza was reported in *Triticum aestivum* by Shahabivand et al. (2012a, b) and Sharma et al. (2016). Similarly, in AM-associated rice roots, the Cd accumulation was less, and the subsequent translocation of Cd into the rice leaves was also decreased. Colonization of *Glomus mosseae* reduced the uptake of Cd to the roots, and it efficiently prevented the xylem transport of the metal to the leaves of *Cajanus cajan* (Garg and Chandel 2012). The mycorrhizae immobilize Cd in the soil with the help of glycoprotein glomalin, produced by the external mycelium, which has metal-binding properties. It was reported that 0.008 mg of Cd was extracted from 1g of glomalin (Gonzalez-Chavez et al. 2004). The fungal hyphae also have a vital role in the chelation of metal ions by improving the passive adsorption of Cd to the hyphae, which will eventually lead to binding of 0.5 mg Cd g^{-1} dry biomass (Joner et al. 2000).

However, in the case of Zn stress, mycorrhization potentially hindered Zn translocation from roots to shoots of rice plants but favored Zn uptake and immobilization in the roots. Similar to our result, Chen and coworkers (Chen et al. 2004a, b) found that mycorrhization reduced foliar metal concentration and increased root Zn content in maize on exposure to 300 mg kg^{-1} of Zn. The increase in the root Zn content is associated with the up-regulation of ZIP transporters. The expression of ZIP transporters increased in the mycorrhizal associated host roots as well as in the fungal mycelium on exposure to Zn toxicity (Ruytinx et al. 2020). Mycorrhizae association decreased the translocation of Zn from soil to the plant body as reported by Christie et al. (2004). Immobilization of high Cd and Zn ions in roots may be considered the major tolerance mechanism of rice plants

towards metal stress, and mycorrhization helps the plant to improve this strategy. The higher TF value for the control (0.81) as compared to the plants subjected to Zn toxicity was due to the low concentration of Zn in the growing media of the control and also due to the inevitability of this essential element in the shoot metabolism. The TF was low (0.18 ± 0.002) in plants exposed to Zn toxicity, and mycorrhization further reduced it to 0.12 ± 0.002. The lower TF value under higher Zn concentration indicates the potential of the plant to reduce the translocation of Zn ions to the metabolically more active shoot regions and thus guard the plants from the adverse effects of metal toxicity (Andrejić et al. 2018).

It was reported that under Zn toxicity, the uptake of Zn by plants could be down-regulated by mycorrhizal colonization (Burleigh et al. 2003). Moreover, fungal vesicles have the potential to chelate Zn ions, and around 300 μg g^{-1} Zn was accumulated in these structures, which was ten times higher than normally reported in plants (Salisbury and Ross 1994).

Conclusion

Heavy metals (Cd and Zn) stress induced drastic and differential metabolic changes in the shoot and root system of rice plants. Developing mycorrhizal association in rice roots helps to enhance the metal tolerance potential by stabilizing Cd in the rhizosphere and Zn in the root tissues where the fungal hyphal ramification was prominent. The mitigation of the oxidative stress elicited by elevated Cd and Zn content with mycorrhization further helps to maintain the changes in the metabolomics and rate of photosynthesis as compared to non-mycorrhizal plants. The results of GC-MS analysis showed the exudation of tributyl acetyl citrate, 3-beta-acetoxystigmasta-4,6,22-triene, and linoleic acid from the mycorrhiza associated roots under heavy metal stress, with strong metal chelation potential aid to keep the toxic metal ions inactive by complexation.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11356-022-22478-y.

Acknowledgements The authors greatly acknowledge the Regional Agricultural Research Station (RARS) of Kerala Agricultural University, Pattambi, Kerala, India, for providing seeds essential to conduct experiments and Centre for Mycorrhizal Culture Collection (CMCC), The Energy and Resources Institute (TERI), New Delhi, for providing the inoculum of mycorrhiza.

Availability of data and materials Data sharing is not applicable to this article as all new created data is already contained within this article

Author contribution E Janeeshma performed the analysis, processed the experimental data, interpreted the results, drafted the manuscript and designed the figures. Jos T. Puthur provided critical feedback and helped shape the research and analysis aided in interpreting the results and worked on the manuscript.

Funding This work was supported by the University Grant Commission (UGC), India, in the form of JRF under grant 319492.

Declarations

Conflict of interest The authors declare no competing interests.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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