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Different responses to manganese toxicity and its mitigation through association with arbuscular mycorrhizal fungus in sunflower and eggplant

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Running title: AMF MITIGATES MN TOXICITY IN SUNFLOWER AND EGGPLANT

Abstract – Manganese (Mn) toxicity is a widespread constraint in plant production; however, limited information is available on the role of arbuscular mycorrhizal fungi (AMF) in enhancing Mn tolerance. In this study, eggplant (*Solanum melongena* L.) and sunflower (*Helianthus annuus* L.) were inoculated with *Rhizophagus intraradices* and exposed to 800 µM Mn for four weeks under greenhouse conditions. Based on biomass production and leaf symptoms, sunflower exhibited greater tolerance to Mn toxicity than eggplant. Mn toxicity symptoms appeared as small black spots associated with leaf trichomes in sunflower, whereas in eggplant, brown spots and speckles developed on the leaf blades without the involvement of stellate trichomes. Under Mn stress, leaf phenolics and anthocyanins increased in sunflower but declined in eggplant. AMF colonisation was higher in sunflower and associated with improved chlorophyll content, enhanced photosynthetic light reactions, and more efficient carbohydrate allocation to the fungal partner compared with eggplant. Although Mn stress reduced root colonisation more strongly in eggplant, biomass production was improved to a similar extent in both species upon AMF inoculation. Photosynthetic light reactions and leaf chlorophyll, carotenoid, phenolic, and macronutrient concentrations were significantly higher under combined Mn toxicity and AMF inoculation than under Mn stress alone, with the effect particularly pronounced in sunflower. The most consistent impact of AMF colonisation was a reduction in Mn accumulation in leaves and roots, likely due to Mn immobilisation in the rhizosphere. Overall, our findings highlight the strong potential of AMF to mitigate Mn toxicity by both reducing Mn accumulation in roots and leaves and enhancing internal detoxification mechanisms.

Keywords: antioxidant enzymes, arbuscular mycorrhizal fungi (AMF), Mn toxicity symptoms, phenolics and anthocyanins

Introduction

Arbuscular mycorrhizal fungi (AMF) are key components of the soil microbial community that form obligate associations with plant roots. The primary benefit of AMF symbiosis is the enhanced supply of phosphorus to host plants. However, AMF also provide several additional functions that are particularly important under stressful growth conditions. Numerous studies have attributed a wide array of beneficial effects to AMF, including mitigation of adverse soil and

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environmental factors, making this mutualistic relationship an essential component of plant adaptive strategies in both natural and agricultural ecosystems (Ahammed and Hajiboland 2024).

Several mechanisms have been proposed to explain how AMF alleviate biotic and abiotic stresses. These include improved water relations, nutrient homeostasis, and photosynthesis, as well as enhanced cellular protection against oxidative stress and increased synthesis of protective metabolites (Begum et al. 2019, Jajoo and Mathur 2021). Root colonisation by AMF increases the plant's nutrient uptake capacity through efficient fungal uptake systems and, in particular, through the extended extraradical hyphae, which enhance spatial access to soil elements (Wipf et al. 2019). Nonetheless, the effect of AMF on nutrient uptake is not universal; it varies depending on the nutrient in question, the plant and fungal species involved, and the prevailing soil and environmental conditions. For instance, in soils with high concentrations of heavy metals, mycorrhizal plants frequently exhibit lower metal accumulation in above-ground tissues than non-mycorrhizal plants. This suggests that AMF can restrict the entry of heavy metals into fungal cells and/or limit their transfer to host plants, thereby playing a critical role in plant resilience to heavy metal–contaminated soils (Hildebrandt et al. 2007, Dhalaria et al. 2020, Riaz et al. 2021).

Manganese (Mn) is an essential micronutrient that plays a central role in photosynthesis as a metal cofactor of the oxygen-evolving complex and functions as a cofactor for enzymes involved in the phenylpropanoid pathway and lipid biosynthesis (Cakmak et al. 2023). However, excessive Mn concentrations are toxic, disrupting cellular processes and impairing plant growth and development (Li et al. 2019, Alejandro et al. 2020, Skórka et al. 2023). Toxic Mn levels are most frequently encountered in acidic soils (pH < 5.5), where solubility increases under low pH conditions. Waterlogging also contributes to Mn toxicity by reducing Mn oxides (MnIV) to the soluble MnII form, thereby elevating root-available Mn^{2+} in soils (Li et al. 2019, Alejandro et al. 2020).

Plant tolerance to Mn toxicity and the manifestation of leaf symptoms vary among species, partly due to differences in Mn distribution within leaves. A common symptom is chlorosis, but its severity and pattern differ across species (Millaleo et al. 2010). Some plants develop necrotic spots (Moroni et al. 2003, Demirevska-Kepova et al. 2004), while others show dark brown (Wissemeier and Horst 1992) or violet (Nezhadasad et al. 2023) speckles. Two models have been proposed to explain these variations: symplastic and apoplastic models (Fernando and Lynch 2015). In the symplastic model, Mn induces photo-oxidative stress in chloroplasts, leading to chlorophyll degradation and chlorosis (Millaleo et al. 2010). In contrast, the apoplastic model attributes leaf lesions to the accumulation of MnO_2 and oxidised phenolics in the apoplast, resulting in necrotic or brown spots (Fecht-Christoffers et al. 2006, Nezhadasad et al. 2023). Thus, species-specific differences in Mn uptake, root-to-shoot transport, and leaf distribution patterns may explain the variable sensitivity of plants to Mn toxicity.

Eggplant (*Solanum melongena* L.; Solanaceae) is an important horticultural crop, widely cultivated for its edible fruits, particularly in Asia and the Mediterranean region. Among solanaceous vegetables, eggplant ranks third in production after potato and tomato (Chapman 2019). Sunflower (*Helianthus annuus* L.; Asteraceae), the fourth most important oil crop globally (Puttha et al. 2023), contributes to nutrient cycling and soil structure improvement due to its vigorous and deep root system. Sunflower is also used in crop rotations as a cover crop and in phytoremediation of heavy metal–contaminated soils (Rizwan et al. 2016).

Sunflower is generally tolerant to Mn toxicity (Schotz et al. 1990). In this species, Mn concentrations of 3–5 mg g^{-1} DW in the aerial parts are sequestered in non-phytotoxic forms,

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primarily as MnO_2 deposits in and around trichomes (Blamey et al. 1986, Hajiboland et al. 2008). By contrast, Mn tolerance and accumulation have not been studied in eggplant.

The ameliorative effects of AMF on heavy metal toxicity have been investigated primarily with cadmium (Cd), followed by lead (Pb), zinc (Zn), and copper (Cu) (Dhalaria et al. 2020, Riaz et al. 2021). Only a few recent studies have examined AMF-mediated alleviation of Mn toxicity, mainly in tree species such as the nutgall tree (*Rhus chinensis*) (Pan et al. 2023), eucalyptus (*Eucalyptus tereticornis*) (De Oliveira et al. 2023), and trifoliate orange (*Poncirus trifoliata*) (Xu et al. 2024). However, much less information is available for field and horticultural crops.

The present study aimed to compare sunflower and eggplant in terms of their tolerance to Mn toxicity and to evaluate potential differences in the role of AMF in mitigating Mn stress between the two species. Such a comparison may provide insights into the mechanisms underlying efficient protection against Mn stress and the species-specific pathways through which AMF confer tolerance. We hypothesised that sunflower and eggplant differ both in their inherent tolerance to Mn toxicity and in their responsiveness to AMF-mediated alleviation of Mn stress.

Materials and methods

Plant cultivation, inoculation, and treatments

Seeds of eggplant (*Solanum melongena* L. cv. Yalda) and sunflower (*Helianthus annuus* L. cv. S300 (601)) were obtained from commercial suppliers. Seeds were surface-sterilised with 1% sodium hypochlorite and germinated in sterile, washed perlite. After leaf emergence, seedlings were irrigated with half-strength Hoagland nutrient solution (pH 5.9) (Johnson et al. 1957).

The arbuscular mycorrhizal fungus *Rhizophagus intraradices* (formerly *Glomus intraradices*), provided by the Soil Science Department, University of Tabriz, was propagated on maize in 5 L pots containing sterile cocopeat:vermiculite (3:1, v/v) under greenhouse conditions. Rorison's nutrient solution (Rorison 1985) with reduced phosphorus (0.5 mM) was supplied twice weekly. After two months, shoots were cut, and the pot contents (roots, spores, and hyphae) were homogenised to serve as inoculum (~50 active propagules g^{-1}).

One-week-old seedlings were transplanted into 1.2 L pots filled with sterile perlite either without inoculum (–AMF) or with inoculum (+AMF). Each pot contained a single plant. The inoculum (40 mg L^{-1} pot) was placed in the planting hole before transplanting. To equalise the physical and microbial conditions with those of +AMF pots, the –AMF pots received both the filtrate (Whatman No. 1) of the inoculum suspension and the autoclaved inoculum. Plants were irrigated with Hoagland solution (pH 5.9) with a modified phosphorus concentration. Phosphorus was maintained at 0.1 mM during the first four weeks to enhance colonisation, then increased to 1.0 mM. The Hoagland solution was supplemented with NH_4Cl to maintain ammonium concentration at 2 mM.

Two weeks after transplanting, pots were assigned randomly to two Mn treatments: (i) control (2.0 µM Mn as in the standard Hoagland solution, sufficient for growth) and (ii) Mn toxicity (800 µM $MnCl_2$). The Mn toxicity level was selected based on a preliminary hydroponic experiment in which plants were exposed to Mn concentrations ranging from 200 to 1200 µM. Distinct growth responses and leaf symptoms were observed at 800 µM, whereas higher concentrations caused severe leaf damage and plant death within one week of treatment. The Mn toxicity treatment was applied gradually by increasing the Mn concentration by 100 µM per day until the final level was reached, using a $MnCl_2$ stock solution (1 mM) supplied through the

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irrigation water. Plants continued to receive nutrient solution following daily weighing, with 250 mL supplied per week and additional water provided on alternate days.

Growth conditions were 25/17 °C (day/night), 14/10 h photoperiod, 50–60% relative humidity, and a photosynthetic photon flux density (PPFD) of ~300 μmol m^{-2} s^{-1} from fluorescent lamps.

Plant harvest

Plants were harvested four weeks after the onset of Mn treatments (six weeks after AMF inoculation). Shoots and roots were separated, rinsed, and blotted dry for fresh weight (FW) determination. Dry weight (DW) was obtained from oven-dried material, which was also used for elemental analysis. A separate set of plants was used for physiological and biochemical assays. All biochemical parameters were determined in the leaves, except for total phenolics, non-structural carbohydrates (soluble sugars and starch), and elemental composition—including Mn and macronutrients (phosphorus, calcium, and potassium)—which were determined in both leaves and roots.

Root colonisation and microscopic observations

Subsamples of fine roots were collected at harvest and used to estimate the colonisation rate. AMF colonisation was quantified using the gridline intersection method (Kormanik et al. 1980). Two subsamples from each replicate pot were analysed, and the mean of four replicate pots was used to calculate mycorrhizal colonisation. Fine roots (~1 g FW) were cleared in 10% KOH, stained with 0.05% trypan blue in lactoglycerol, and examined under a stereomicroscope.

For Mn localisation, chlorophyll and phenolics were removed by immersing leaves in boiling ethanol (98%, v/v) for 3 h. Leaves were photographed before and after clearing (Nezhadasad et al. 2023).

Chlorophyll fluorescence parameters and pigments

Photosynthetic light reactions were assessed by measuring chlorophyll (Chl) fluorescence parameters using a fluorometer (OSF1, ADC Bioscientific, UK). The maximum quantum yield of photosystem II (PSII) (F_v/F_m) and maximum primary yield of PSII (F_v/F_0) were measured in dark-adapted leaves. In light-adapted leaves, excitation capture efficiency (F_v'/F_m'), photochemical quenching (qP), and nonphotochemical quenching (qN) were calculated (Maxwell and Johnson 2000).

Chlorophyll and carotenoids (Car) were extracted by homogenising ~0.1 g of freshly harvested leaves in 1 mL of 80% acetone at 4 °C in the dark. Absorbance was recorded at 646, 663, and 470 nm, and pigment contents were calculated on a fresh weight basis (Lichtenthaler and Wellburn 1983).

Leaf anthocyanin content was determined using the pH differential method described by Tonutare et al. (2014). Approximately 0.1 g of freshly harvested leaves was extracted in 1 mL of methanol containing 2% HCl (v/v), and the resulting homogenate was centrifuged at 5000 g. The supernatant was then diluted (1:2) with two buffer solutions: 25 mM KCl (pH 1.0) and 400 mM sodium acetate (pH 4.5). Absorbance was measured at 510 nm and 700 nm, and anthocyanin content was calculated as cyaniding-3-O-glucoside equivalents using an extinction coefficient of 26.9 mM^{-1} cm^{-1} (Tonutare et al. 2014).

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Oxidative stress markers and antioxidant defence enzymes

The content of H_2O_2 was determined following the potassium iodide (KI) method described by Junglee et al. (2014). Fresh leaf tissue was homogenised in 0.1% (w/v) trichloroacetic acid (TCA) at a ratio of 0.1 g fresh weight per 1 mL TCA and centrifuged. For the colorimetric reaction, 250 µL of the extract supernatant was mixed with 10 mM potassium phosphate buffer (pH 7.0) and 500 mM KI. The mixture was incubated in the dark for 15 min at room temperature, and absorbance was measured at 390 nm. The H_2O_2 content was calculated from a standard curve prepared with known H_2O_2 concentrations and expressed on a fresh weight basis (Junglee et al. 2014).

Lipid peroxidation was estimated from the amount of malondialdehyde (MDA) formed in the reaction mixture. Leaf tissues were homogenised in 0.1% (w/v) TCA at a ratio of 0.1 g fresh weight per 1 mL TCA. The reaction mixture consisted of 1 mL supernatant and 4 mL of 20% (w/v) TCA containing 0.5% (w/v) thiobarbituric acid (Sigma). The mixture was heated at 95 °C for 30 min and then quickly cooled in ice. After centrifugation at 10 000 g for 15 min, the absorbance of the supernatant was measured at 532 nm. MDA levels were calculated using a 1,1,3,3-tetraethoxypropane (Sigma) standard curve (Hodges et al. 1999).

Activity of antioxidant defence enzymes was determined using fresh leaf samples. Approximately 0.1 g of leaf tissue was ground in an extraction buffer (0.1 g FW per 1 mL buffer) using a pre-chilled mortar and pestle, and the homogenate was centrifuged at 10 000 g for 10 min at 4 °C. The supernatant was used for enzyme assays. For each enzyme, the assay was tested for linearity between the volume of crude extract and the measured activity. Changes in absorbance of the substrate or product (depending on the assay protocol) were monitored using a spectrophotometer (Specord 200, Analytik Jena, Germany). Enzyme activities were calculated on a protein content basis and expressed as specific activity. Total soluble protein was quantified using Bradford reagent (Sigma) with bovine serum albumin (BSA) as the standard.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined according to Giannopolitis and Ries (1977). The enzyme was extracted in a buffer containing 25 mM HEPES (pH 7.8) and 0.1 mM EDTA, and the homogenate was centrifuged. The reaction mixture consisted of 25 µL enzyme extract and 450 µL reaction medium containing 25 mM HEPES (pH 7.6), 0.1 mM EDTA, 50 mM Na_2CO_3 (pH 10.2), 12 mM L-methionine, 75 µM NBT (nitro blue tetrazolium chloride), and 1 µM riboflavin. The reaction mixtures were incubated at 22 °C under a light intensity of 400 µmol m^{-2} s^{-1} for 10 min. A control sample without enzyme extract was included. The absorbance of the blue formazan formed as a result of NBT reduction was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT reduction compared with the control sample without enzyme extract (Giannopolitis and Ries 1977).

Catalase (CAT, EC 1.11.1.6) activity was assayed by monitoring the decrease in absorbance of H_2O_2. The enzyme was extracted in 50 mM phosphate buffer (pH 7.0). The assay mixture contained 50 mM phosphate buffer and 10 mM H_2O_2. The reaction was initiated by adding 100 µL of enzyme extract to the reaction mixture, and the decrease in absorbance of H_2O_2 at 240 nm was monitored for 3 min. Enzyme activity was calculated using an extinction coefficient for H_2O_2 of 0.041 mM^{-1} cm^{-1} and expressed as µmol H_2O_2 mg^{-1} protein min^{-1} (Aebi 1984).

Ascorbate peroxidase (APX, EC 1.11.1.11) was extracted in 50 mM phosphate buffer (pH 7.0), and 100 µL of the supernatant was used for the enzyme assay. The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 0.2 mM EDTA, 0.5 mM ascorbic acid, 50 mg BSA, and 100 µL crude enzyme extract. The reaction was initiated by adding H_2O_2 to a final

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concentration of 0.1 mM. Oxidation of ascorbic acid was monitored as a decrease in absorbance at 290 nm for 3 min after starting the reaction. Enzyme activity was calculated using an extinction coefficient for ascorbic acid of 2.8 mM^{-1} cm^{-1} and expressed as µmol ascorbic acid mg^{-1} protein min^{-1} (Nakano and Asada 1981).

Total soluble phenolics and phenolic-metabolising enzymes

Total free (soluble) phenolics were extracted from 0.3 g of freshly harvested leaves and roots using 70% methanol at 4 °C in the dark. The extraction was performed three times, each with 1 mL of methanol, and the extracts from the three successive steps were pooled. After centrifugation, the supernatant was analysed using the Folin–Ciocalteu reagent. A standard curve was prepared with gallic acid, and the total phenolic content was expressed as gallic acid equivalents (Blainski et al. 2013).

Guaiacol peroxidase (POD, EC 1.11.1.7) was extracted in 10 mM phosphate buffer (pH 7.0). After centrifugation, the enzyme activity was assayed in a reaction mixture containing 10 mM phosphate buffer, 5 mM H_2O_2, and 4 mM guaiacol. The reaction was initiated by adding 100 µL crude enzyme extract at 25 °C. After 2 min, the formation of tetraguaiacol was monitored for 3 min at 470 nm. Enzyme activity was expressed as µmol tetraguaiacol mg^{-1} protein min^{-1} (Chance and Maehly 1955).

Phenylalanine ammonia lyase (PAL, EC 4.3.1.5) activity was determined as the amount of *trans*-cinnamic acid formed from L-phenylalanine (Jun et al. 2018). The enzyme was extracted in a medium containing 100 mM borate buffer, 1.4 mM mercaptoethanol, and 9 mM PVPP. After centrifugation, the assay mixture contained borate buffer (pH 8.8), 12 mM L-phenylalanine, and 100 µL crude enzyme extract. The mixture was incubated at 30 °C for 30 min. After centrifugation, the absorbance of *trans*-cinnamic acid was measured at 290 nm, and enzyme activity was calculated using its extinction coefficient (9.63 mM^{-1} cm^{-1}) (Jun et al. 2018).

Polyphenol oxidase (PPO, EC 1.14.18.1) was extracted in 200 mM phosphate buffer (pH 6.5). After centrifugation, 50 µL of crude enzyme extract was mixed with 200 mM phosphate buffer (pH 6.5) and 10 mM pyrogallol. The reaction mixture was incubated for 3 min at 38 °C. Enzyme activity was determined from the change in absorbance at 334 nm for 3 min due to pyrogallol oxidation and expressed as ΔAbs mg^{-1} protein min^{-1} (Casado-Vela et al. 2005).

Non-structural carbohydrates

For the determination of non-structural carbohydrates (soluble sugars and starch), fresh leaf and root samples were homogenised in 96% (v/v) ethanol at 4 °C (0.1 g FW per 1 mL ethanol). After centrifugation at 12 000 g for 15 min, the supernatant was used to determine total soluble sugars using the anthrone–sulfuric acid method with glucose as a standard. The remaining pellet was mixed with 1 mL of 80% dimethyl sulfoxide (DMSO) containing 8 N HCl (DMSO:HCl, 8:2 v/v) and incubated at 60 °C for 30 min to dissolve starch. After centrifugation, the supernatant was used for starch determination using freshly prepared Lugol's reagent (I_2–KI), composed of 2% (w/v) KI and 0.2% (w/v) I_2. The reaction mixture was incubated for 10 min at room temperature, and absorbance was measured spectrophotometrically at 620 nm. A standard curve was prepared using maize starch (Maness 2010).

Macronutrients and Mn

Dried leaf and root samples were dry ashed at 500 °C for 8 h, and residues were dissolved in 10% HCl. Extracts were diluted to volume with deionised water. Phosphorus (P) concentration

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was determined spectrophotometrically using ammonium vanadate molybdate reagent, while calcium (Ca) and potassium (K) were measured using flame photometry (PFP7, Jenway, UK). Concentration of Mn was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES; ICP5000DV, PG Instruments Ltd.).

Experimental design and statistical analysis

The experiment was conducted using a completely randomised design with four independent pots serving as replicates. Pairwise comparisons of means (one-way ANOVA) for each parameter within each plant species were performed using Tukey's test ($P < 0.05$). In addition, a three-way ANOVA including the main factors (species, Mn treatment, and AMF inoculation) was performed for all biochemical and elemental data using SigmaStat 3.5. In some cases, an additional t-test was performed ($P < 0.05$) to detect differences between the two Mn levels within the AMF treatment that were not revealed by pairwise comparisons among the four treatment groups.

Results

In this study, significant effects of species, Mn treatment, and AMF inoculation, as well as several interactions among these factors, were detected for most biochemical and elemental parameters according to the three-way ANOVA (On-line Suppl. Tab. 1). The significant species-specific effect observed for many variables indicates clear differences between eggplant and sunflower in their physiological and nutritional responses to Mn toxicity and mycorrhizal colonisation.

Colonisation rate and plant biomass production

Root colonisation rates (%) were significantly higher in sunflower (88–94%) than in eggplant (53–75%) under both control ($P < 0.05$) and Mn toxicity conditions ($P < 0.05$) (Fig. 1a, b, On-line Suppl. Tab. 1). Exposure to excessive Mn caused a more pronounced reduction in root colonisation in eggplant (29.4%) compared with sunflower (5.5%) (Fig. 1a, b, On-line Suppl. Tab. 1).

Under control conditions, shoot and root dry biomass were significantly increased by mycorrhizal colonisation in eggplant. Mn toxicity significantly reduced both shoot and root dry biomass in this species. The reduction was more pronounced in the shoot, with a 55% decrease compared with a 31% reduction in the root. Inoculation with AMF substantially enhanced shoot and root biomass in eggplant under Mn toxicity conditions (Fig. 1c, e).

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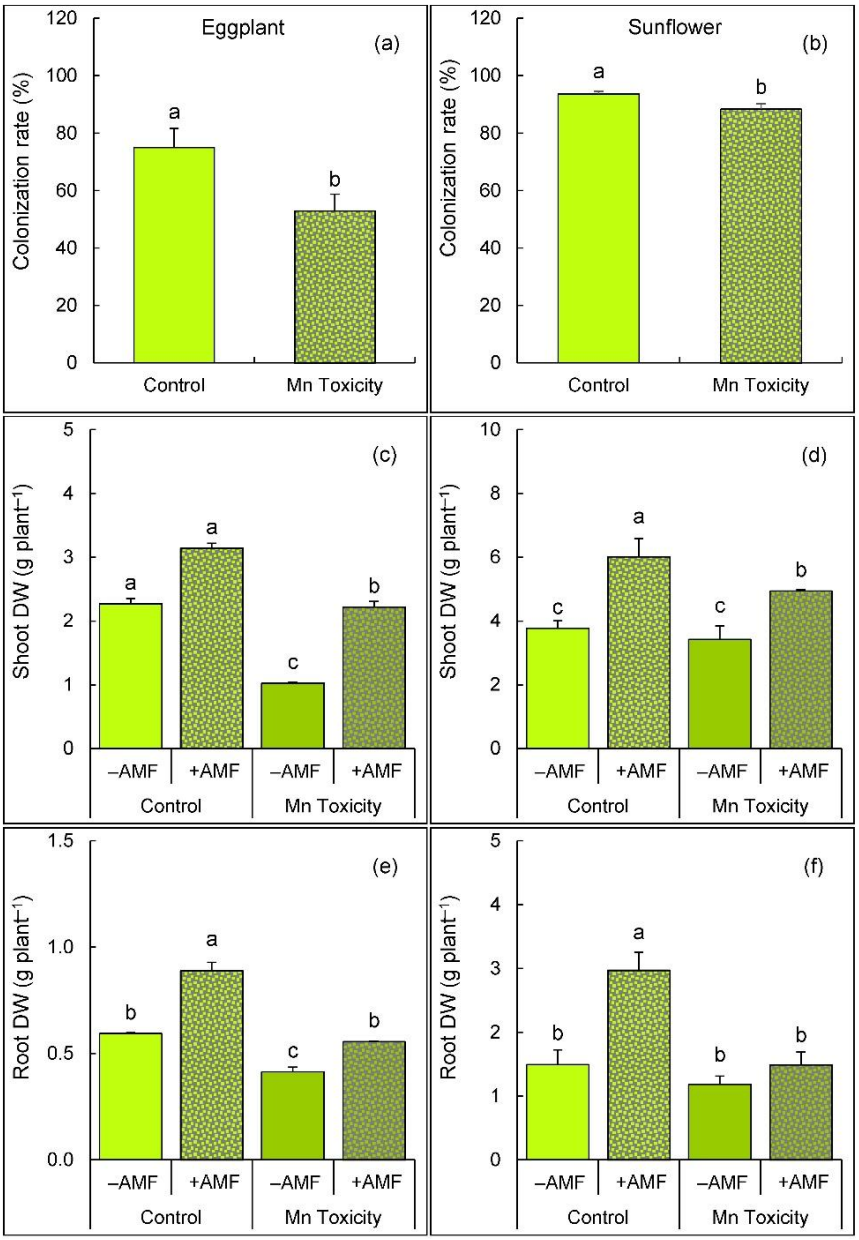

Fig. 1. Effects of arbuscular mycorrhizal fungus (*Rhizophagus intraradices*) and Mn toxicity (800 µM $MnCl_2$) on root colonisation and growth of eggplant (*Solanum melongena* L.) and sunflower (*Helianthus annuus* L.). Root colonisation rate (%) (a, b), shoot dry weight (DW) (c, d) and root DW (e, f) in non-inoculated (–AMF) and inoculated (+AMF) plants. Data are means (n = 4) ± SD. Differences among data indicated by different letters are statistically significant ($P < 0.05$).

In sunflower, mycorrhizal inoculation significantly increased shoot and root biomass under control conditions. Mn toxicity, however, did not significantly influence either shoot or root dry biomass in this species. Under Mn stress conditions, mycorrhizal inoculation also increased biomass, with the effect being significant for shoot dry weight (Fig. 1d, f).

Leaf symptoms

In eggplant, the first visible symptoms of Mn toxicity appeared two weeks after the onset of treatment. Chlorosis and light-brown speckles developed on the leaf blade, which later progressed to necrotic lesions (Fig. 2a). Treatment with hot ethanol, conducted to remove leaf pigments, revealed numerous dark-brown spots distributed throughout the leaf blade (Fig. 2b). These spots showed no specific association with trichomes (Fig. 2c). Leaf cross-sections confirmed that the black coloration observed at the base of stellate trichomes was also present in control plants (Fig. 2d), indicating that these sites are not associated with accumulation of oxidised Mn.

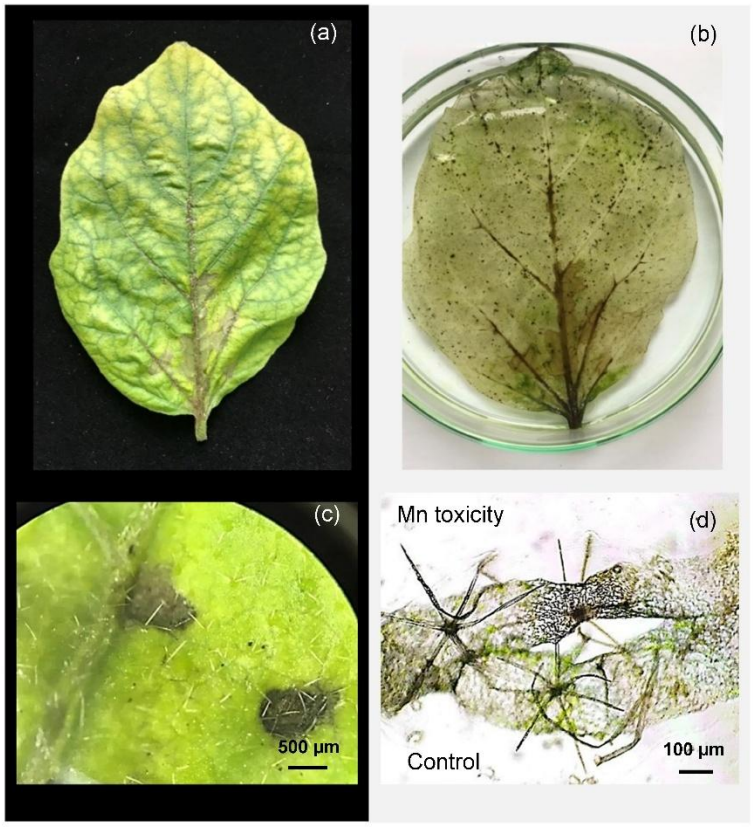

Fig. 2. Leaf symptoms and anatomical changes in eggplant (*Solanum melongena* L.) exposed to Mn toxicity (800 µM $MnCl_2$) for four weeks. Leaf symptoms before (a) and after hot ethanol treatment (b), appearance of brown speckles (c), and leaf cross-sections showing Mn toxicity (top) and control plants (bottom) (d).

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In sunflower, the primary symptom of Mn toxicity was the formation of black spots at the base of leaf trichomes (Fig. 3a), which became more prominent following hot ethanol treatment (Fig. 2b). These spots appeared on leaf blades, petioles (Fig. 3c), and, to a lesser extent, on the stem. Necrotic lesions occasionally developed on older leaves under prolonged Mn exposure (Fig. 3a). In both species, particularly in sunflower, symptoms were less severe and appeared later in mycorrhizal (+AMF) plants compared with non-mycorrhizal (–AMF) plants (data not shown). However, the spatial pattern of speckles and necrotic spots did not differ between –AMF and +AMF plants.

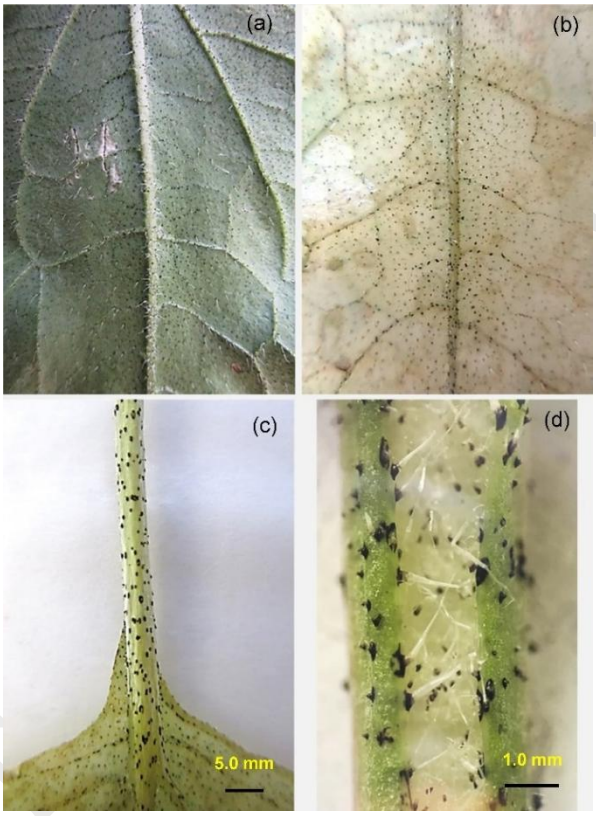

Fig. 3. Leaf symptoms and anatomical changes in sunflower (*Helianthus annuus* L.) exposed to Mn toxicity (800 µM $MnCl_2$) for four weeks. Leaf symptoms before (a) and after hot ethanol treatment (b), appearance of black spots (c), and their localisation at the base of trichomes (d).

Leaf pigment content and photochemical parameters

In the absence of excess Mn, AMF inoculation decreased leaf chlorophyll (Chl *a*+*b*) content in eggplant but increased it in sunflower. Leaf chlorophyll content was significantly reduced under Mn toxicity, with the effect being more pronounced in eggplant (Tab. 1). Under Mn stress, AMF inoculation enhanced leaf Chl levels in both species (Tab. 1).

Leaf Car content exhibited a similar response to AMF and Mn treatments in both species: it increased with mycorrhizal colonisation under control conditions but decreased under excess Mn. Similar to Chl *a*+*b*, leaf Car content under Mn toxicity conditions was increased by AMF inoculation in both species (Tab. 1).

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Leaf anthocyanin content significantly decreased in response to AMF inoculation in eggplant but remained unaffected in sunflower under control conditions. Under Mn stress, leaf anthocyanin content responded differently in the two species: it decreased in eggplant but increased up to 3.2-fold in sunflower (Tab. 1). AMF inoculation in Mn-stressed plants decreased leaf anthocyanin content in sunflower but did not affect it in eggplant (Tab. 1).

Tab. 1. Contents of chlorophylls (Chl *a+b*) (mg g^{-1} FW), carotenoids (µg g^{-1} FW) and anthocyanins (µg cyanidine-3-glucoside g^{-1} FW) in leaves of eggplant (*Solanum melongena* L.) and sunflower (*Helianthus annuus* L.) grown without arbuscular mycorrhizal fungus (–AMF) or inoculated with *Rhizophagus intraradices* (+AMF) under control or Mn toxicity conditions (800 µM as $MnCl_2$) for four weeks. Data are means (n = 4) ± SD. Differences among data of each species indicated by different letters are statistically significant ($P < 0.05$).

Species	Treatments		Chl (*a+b*)	Carotenoids	Anthocyanins
Eggplant	Control	–AMF	5.05±0.07 [a]	222±23 [b]	54±8 [a]
		+AMF	4.52±0.10 [b]	337±40 [a]	32±3 [b]
	Mn toxicity	–AMF	1.97±0.32 [c]	118±22 [c]	25±5 [b]
		+AMF	5.24±0.14 [a]	230±27 [b]	21±10 [b]
Sunflower	Control	–AMF	1.68±0.11 [c]	206±26 [b]	117±3 [b]
		+AMF	2.40±0.11 [b]	370±12 [a]	108±10 [b]
	Mn toxicity	–AMF	1.35±0.11 [d]	74±17 [d]	375±32 [a]
		+AMF	3.48±0.20 [a]	155±18 [c]	138±41 [b]

Mycorrhizal colonisation under control conditions had no significant effect on F_v/F_m, F_v/F_0, F_v'/F_m', or qP, whereas Mn toxicity significantly decreased these parameters in eggplant. In sunflower, in contrast, AMF treatment significantly increased F_v/F_m, F_v/F_0, F_v'/F_m', and qP under control conditions, whereas these parameters remained unaffected under excess Mn. In both species, AMF inoculation in Mn-stressed plants significantly increased these parameters. This effect restored them to the level of –AMF control plants in eggplant, while in sunflower the values were higher than those of –AMF control plants (Tab. 2).

AMF inoculation in control plants enhanced qN in eggplant, whereas no significant effect was observed in sunflower. Mn stress did not significantly affect qN in either species, but AMF inoculation of Mn-stressed plants increased this parameter, with the effect being significant in eggplant (Tab. 2).

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Tab. 2. Chlorophyll fluorescence parameters including maximum quantum efficiency of PSII (F_v/F_m), maximum primary yield of PSII (F_v/F_0), actual quantum efficiency of PSII (F_v'/F_m'), photochemical quenching (qP) and non-photochemical quenching (qN) in leaves of eggplant (*Solanum melongena* L.) and sunflower (*Helianthus annuus* L.) grown without arbuscular mycorrhizal fungus (–AMF) or inoculated with *Rhizophagus intraradices* (+AMF) under control or Mn toxicity conditions (800 µM as $MnCl_2$) for four weeks. Data are means (n = 4) ± SD. Differences among data of each species indicated by different letters are statistically significant ($P < 0.05$).

Species	Treatments		F_v/F_m	F_v/F_0	F_v'/F_m'	qP	qN
Eggplant	Control	–AMF	0.83±0.01^{a}	4.80±0.27^{a}	0.75±0.02^{ab}	0.82±0.02^{ab}	0.43±0.02^{c}
		+AMF	0.81±0.02^{a}	4.46±0.24^{a}	0.70±0.05^{b}	0.81±0.03^{ab}	0.49±0.03^{b}
	Mn toxicity	–AMF	0.72±0.01^{b}	3.49±0.17^{b}	0.62±0.02^{c}	0.78±0.02^{b}	0.32±0.02^{c}
		+AMF	0.83±0.01^{a}	5.05±0.46^{a}	0.77±0.01^{a}	0.85±0.02^{a}	0.54±0.03^{a}
Sunflower	Control	–AMF	0.78±0.02^{b}	3.23±0.28^{b}	0.66±0.02^{b}	0.90±0.01^{b}	0.48±0.02^{b}
		+AMF	0.82±0.01^{a}	4.78±0.33^{a}	0.74±0.01^{a}	0.96±0.03^{a}	0.49±0.03^{b}
	Mn toxicity	–AMF	0.78±0.01^{b}	3.80±0.43^{b}	0.68±0.02^{b}	0.92±0.04^{ab}	0.51±0.02^{ab}
		+AMF	0.83±0.01^{a}	4.74±0.26^{a}	0.73±0.02^{a}	0.94±0.01^{ab}	0.54±0.02^{a}

Levels of oxidant metabolites and activity of antioxidant defence enzymes

Under control conditions, leaf H_2O_2 content decreased in response to AMF inoculation in both species, with the reduction being significant in sunflower. Mn toxicity did not influence leaf H_2O_2 content in eggplant but increased it in sunflower. In both species, the leaf H_2O_2 content of Mn-stressed plants significantly decreased upon mycorrhisation (Fig. 4a, b).

Colonisation with AMF decreased leaf MDA content in eggplant but increased it in sunflower under control conditions. Mn toxicity increased leaf MDA content in both species. AMF inoculation in Mn-stressed plants significantly decreased leaf MDA level in eggplant but, similar to control plants, increased it in sunflower (Fig. 4c, d).

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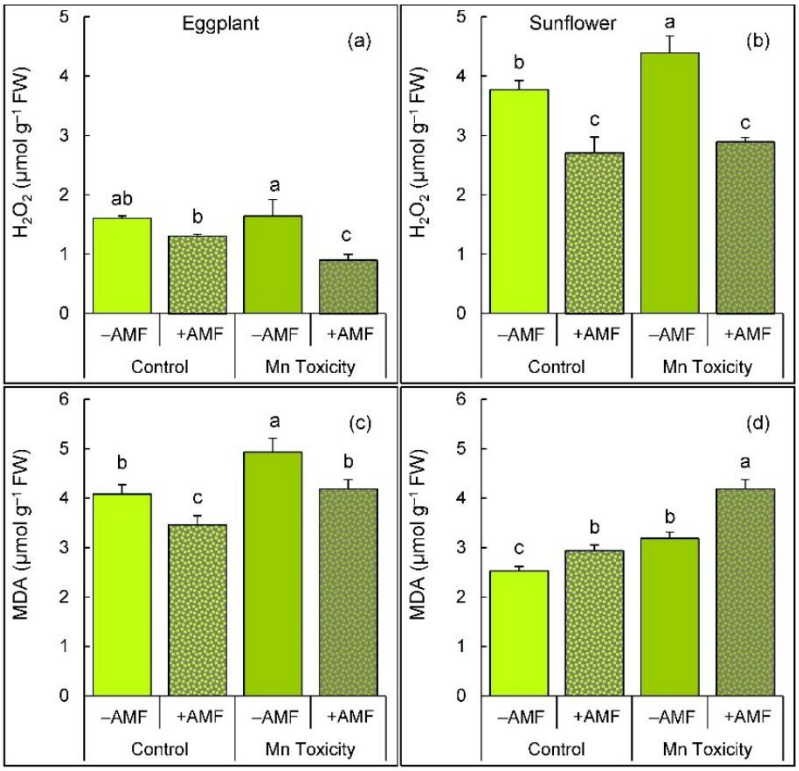

Fig. 4. Contents of hydrogen peroxide (H_2O_2) (a, b) and malondialdehyde (MDA) (c, d) in leaves of eggplant (*Solanum melongena* L.) and sunflower (*Helianthus annuus* L.) grown without arbuscular mycorrhizal fungus (–AMF) or inoculated with *Rhizophagus intraradices* (+AMF) under control or Mn toxicity conditions (800 µM as $MnCl_2$) for four weeks. Data are means (n = 4) ± SD. Differences among data indicated by different letters are statistically significant ($P < 0.05$).

Leaf SOD activity was not affected by AMF in either species under control conditions. Mn toxicity decreased this parameter by up to 5.3-fold in eggplant but slightly increased it in sunflower. In Mn-stressed plants, AMF inoculation tended to reduce leaf SOD activity in eggplant but slightly increased it in sunflower. Consequently, in +AMF plants, SOD activity was significantly higher under Mn stress than under control conditions in sunflower, whereas in eggplant it was up to 14.8-fold lower than in the control plants (Fig. 5a, b).

Under control conditions, AMF inoculation significantly reduced leaf CAT activity in eggplant but had no effect on sunflower. While Mn toxicity did not alter CAT levels in eggplant, it induced a significant increase in sunflower. Notably, AMF inoculation under Mn-stress conditions resulted in no significant changes to CAT activity for either species (Fig. 5c, d).

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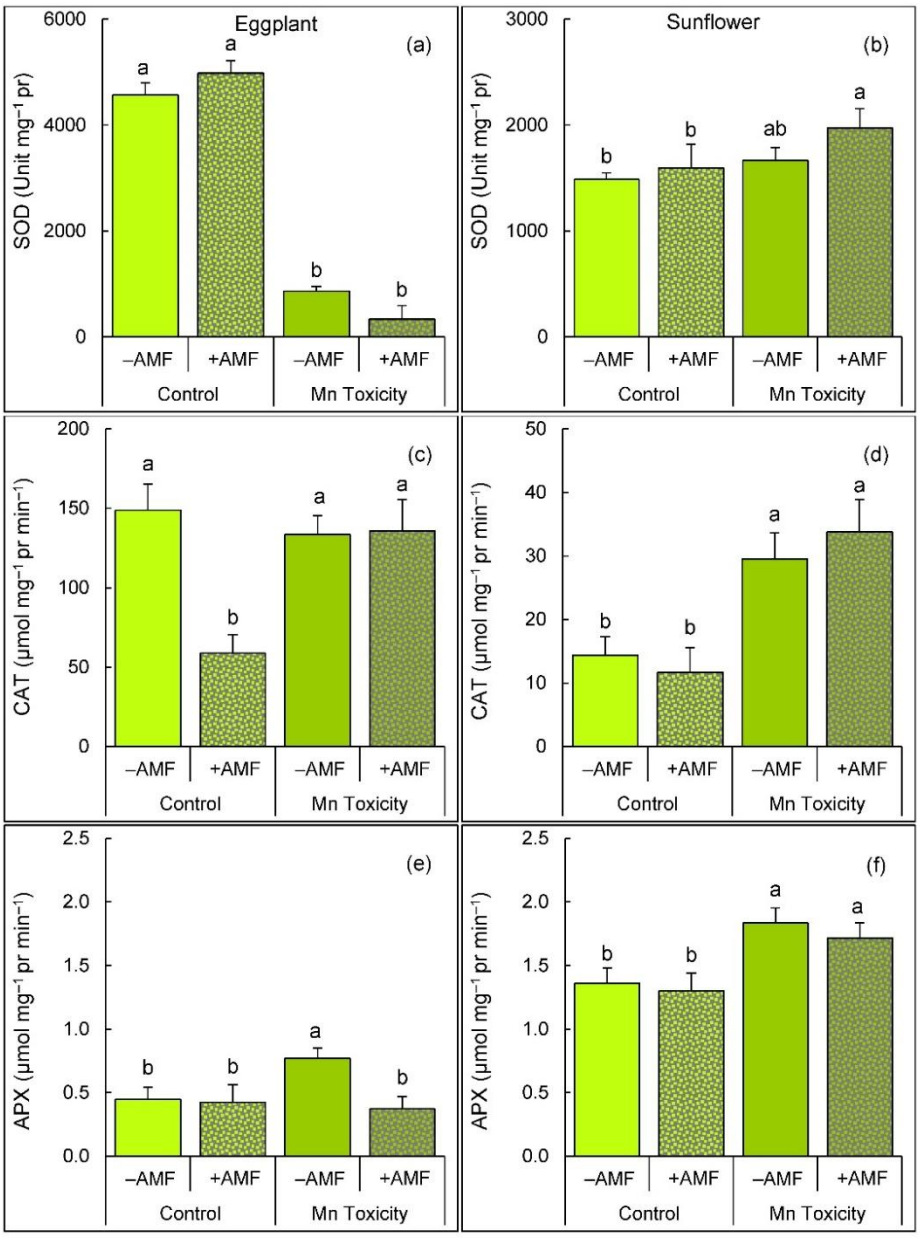

Fig. 5. Activity of superoxide dismutase (SOD) (a, b), catalase (CAT) (c, d) and ascorbate peroxidase (APX) (e, f) in leaves of eggplant (*Solanum melongena* L.) and sunflower (*Helianthus annuus* L.) grown without arbuscular mycorrhizal fungus (–AMF) or inoculated with *Rhizophagus intraradices* (+AMF) under control or Mn toxicity conditions (800 µM as $MnCl_2$) for four weeks. Data are means (n = 4) ± SD. Differences among data indicated by different letters are statistically significant ($P < 0.05$).

Leaf APX activity was not influenced by AMF under control conditions but increased significantly in both species under Mn toxicity (Fig. 5e, f). In Mn-stressed plants, AMF colonisation maintained elevated APX activity in sunflower, whereas in eggplant it reduced the activity to levels comparable to those of the control plants (Fig. 5e, f).

Phenolics content and activity of phenolic-metabolising enzymes

Total soluble phenolics in eggplant leaves decreased when either AMF inoculation or Mn toxicity was applied alone. Under the combined treatment, leaf phenolics increased, though remaining lower than in non-inoculated control plants (Fig. 6a). In sunflower, AMF alone reduced leaf total phenolics content, whereas Mn toxicity increased it, and the combination of Mn and AMF led to a further rise in leaf total phenolics content (Fig. 6b).

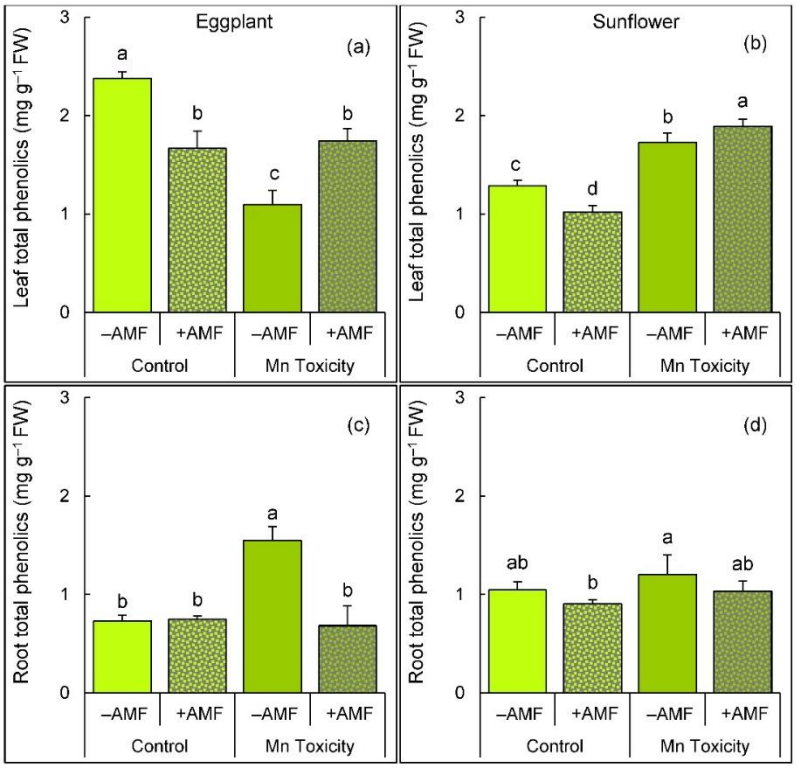

Fig. 6. Contents of total soluble phenolics in leaves (a, b) and roots (c, d) of eggplant (*Solanum melongena* L.) and sunflower (*Helianthus annuus* L.) grown without arbuscular mycorrhizal fungus (–AMF) or inoculated with *Rhizophagus intraradices* (+AMF) under control or Mn toxicity conditions (800 µM as $MnCl_2$) for four weeks. Data are means (n = 4) ± SD. Differences among data indicated by different letters are statistically significant ($P < 0.05$).

In eggplant roots, AMF inoculation had no significant effect on total phenolics content under control conditions, whereas Mn toxicity increased this parameter. AMF inoculation in Mn-stressed plants significantly decreased root total phenolics to the level observed in control plants (Fig. 6c). In sunflower roots, total phenolics content was not significantly influenced by any treatment (Fig. 6d).

Leaf POD activity was not affected by AMF inoculation in control plants but slightly increased under Mn toxicity in eggplant. AMF inoculation in Mn-stressed plants slightly decreased leaf POD activity in this species (Fig. 7a). In sunflower, leaf POD activity was slightly increased by either AMF inoculation or excess Mn when applied individually, but this effect was not observed under the combined treatment (Fig. 7b).

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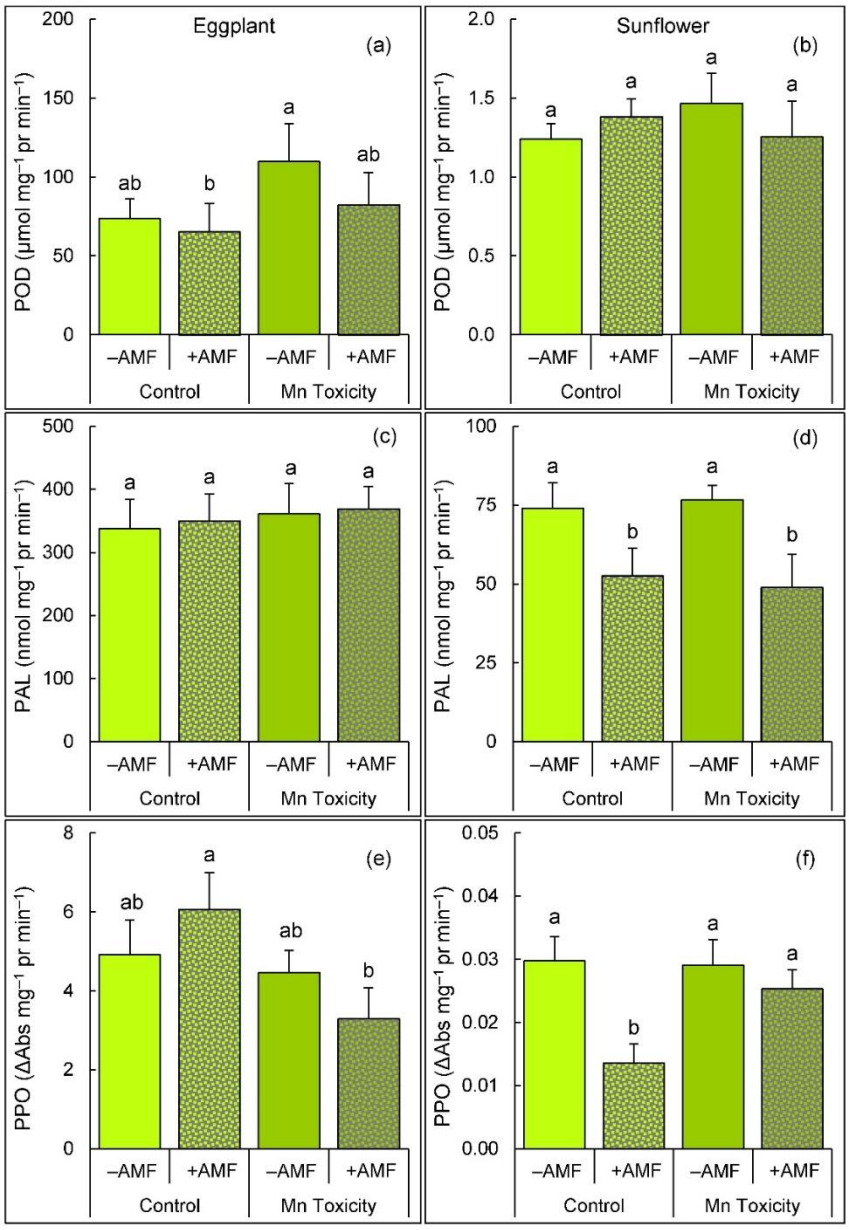

Fig. 7. Activity of peroxidase (POD) (a, b), phenylalanine ammonia lyase (PAL) (c, d) and polyphenol oxidase (PPO) (e, f) in leaves of eggplant (*Solanum melongena* L.) and sunflower (*Helianthus annuus* L.) grown without arbuscular mycorrhizal fungus (–AMF) or inoculated with *Rhizophagus intraradices* (+AMF) under control or Mn toxicity conditions (800 μM as $MnCl_2$) for four weeks. Data are means (n = 4) ± SD. Differences among data indicated by different letters are statistically significant ($P < 0.05$).

Leaf PAL activity was unaffected by any treatments in eggplant, whereas in sunflower AMF inoculation significantly decreased PAL activity under both control and Mn toxicity conditions (Fig. 7c, d).

In eggplant, leaf PPO activity slightly increased following AMF inoculation under control conditions but showed no response to Mn toxicity. Under Mn stress, AMF slightly decreased PPO

activity, resulting in significantly lower levels in +AMF plants compared with the control counterparts (Fig. 7e). In sunflower, AMF significantly reduced leaf PPO activity under control conditions. PPO activity remained unchanged under Mn stress in this species, and unlike in the control treatment, AMF inoculation had no effect under Mn toxicity (Fig. 7f).

Carbohydrate levels

Under control conditions, AMF colonisation significantly reduced leaf soluble sugar and starch contents in eggplant, whereas it increased both parameters in sunflower. Mn toxicity significantly decreased leaf carbohydrate contents, while AMF inoculation in Mn-stressed plants substantially increased these parameters in both species (Tab. 3).

Tab. 3. Contents of soluble sugars and starch (mg g^{-1} FW) in leaves and roots of eggplant (*Solanum melongena* L.) and sunflower (*Helianthus annuus* L.) grown without arbuscular mycorrhizal fungus (–AMF) or inoculated with *Rhizophagus intraradices* (+AMF) under control or Mn toxicity conditions (800 µM as $MnCl_2$) for four weeks. Data are means (n = 4) ± SD. Differences among data of each species indicated by different letters are statistically significant ($P < 0.05$).

Species	Treatments		Leaves		Roots	
			Soluble sugars	Starch	Soluble sugars	Starch
Eggplant	Control	–AMF	34±1.9^{a}	0.62±0.03^{a}	13±1.2^{a}	0.10±0.01^{a}
		+AMF	21±1.4^{b}	0.49±0.03^{b}	6.4±0.3^{c}	0.12±0.03^{a}
	Mn toxicity	–AMF	18±0.7^{c}	0.41±0.03^{b}	9.3±0.7^{b}	0.02±0.00^{b}
		+AMF	22±0.7^{b}	0.62±0.01^{a}	6.8±0.5^{c}	0.11±0.03^{a}
Sunflower	Control	–AMF	58±1. ^{b}	0.66±0.04^{c}	43±2.9^{b}	0.09±0.01^{b}
		+AMF	76±5.7^{a}	0.98±0.06^{b}	50±2.0^{a}	0.15±0.02^{a}
	Mn toxicity	–AMF	39±3.2^{c}	0.30±0.02^{d}	32±4.7^{c}	0.08±0.02^{b}
		+AMF	56±6.5^{b}	1.10±0.07^{a}	49±2.3^{ab}	0.09±0.01^{b}

In the roots, AMF colonisation of control plants decreased soluble sugar content in eggplant but increased both soluble sugar and starch contents in sunflower, similar to that observed in the leaves of this species. A similar trend to that of the leaves was observed for the effect of Mn toxicity on root carbohydrates: reductions in both species except for starch in sunflower. AMF inoculation in Mn-stressed plants affected root carbohydrate levels differently in the two species: in eggplant it decreased soluble sugar but increased starch content, whereas in sunflower it increased soluble sugar with no change in starch (Tab. 3).

Macronutrients and Mn concentrations

Inoculation with AMF did not affect leaf Mn concentration in either species but decreased root Mn concentration in eggplant (Fig. 8). As expected, Mn concentrations in both leaves and roots increased under excess Mn in both species (Fig. 8). AMF inoculation significantly reduced Mn accumulation in the leaves and roots of Mn-stressed plants. This reduction was more pronounced in sunflower than in eggplant, particularly in the leaves (Fig. 8).

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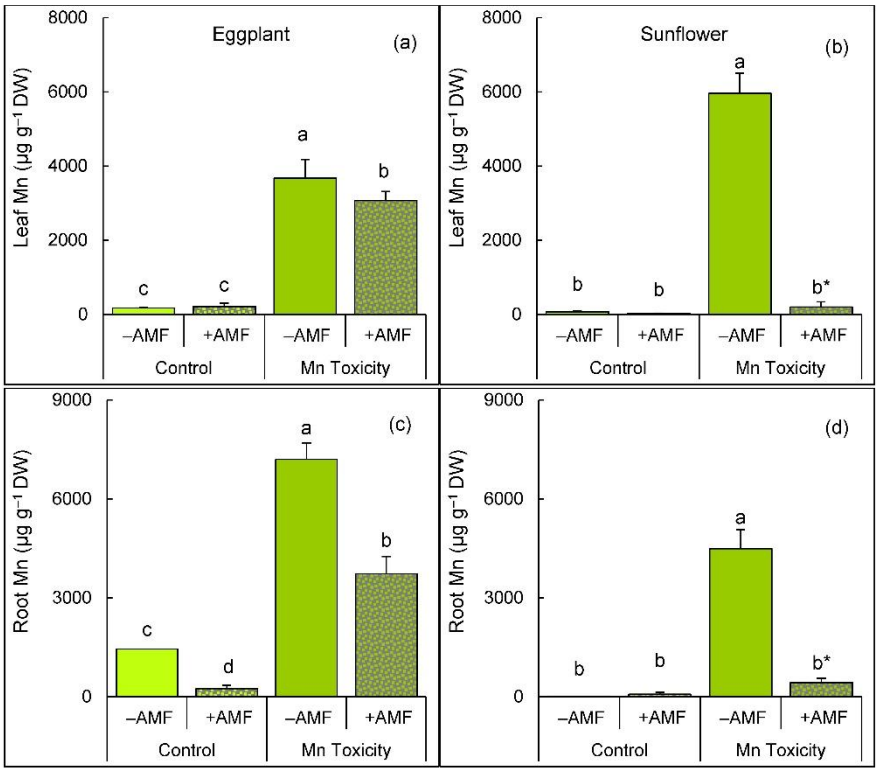

Fig. 8. Concentrations of Mn in leaves (a, b) and roots (c, d) of eggplant (*Solanum melongena* L.) and sunflower (*Helianthus annuus* L.) grown without arbuscular mycorrhizal fungus (–AMF) or inoculated with *Rhizophagus intraradices* (+AMF) under control or Mn toxicity conditions (800 µM as $MnCl_2$) for four weeks. Data are means (n = 4) ± SD. Differences among data indicated by different letters are statistically significant ($P < 0.05$). Asterisks denote a significant difference compared with control plants.

Under control conditions, AMF inoculation increased leaf and root P concentration in eggplant but did not affect this parameter in sunflower leaves or roots (Tab. 4). In eggplant, leaf and root P concentrations were higher under Mn toxicity compared with control plants, whereas in sunflower this effect was not significant. AMF inoculation under Mn stress conditions led to significantly higher P concentration in the leaves of both species, whereas P concentration in the roots remained unaffected in either species (Tab. 4).

Ca concentration was not affected by AMF inoculation under control conditions either in leaves or roots, except for a significant reduction in eggplant roots. Mn toxicity increased Ca concentration in leaves and roots of eggplant, whereas in sunflower it increased Ca concentration in the leaves but decreased it in the roots (Tab. 4). Under Mn stress, AMF inoculation decreased leaf Ca concentration in both species, with the effect being significant in eggplant. In the roots, however, AMF decreased Ca concentration in eggplant but increased it in sunflower (Tab. 4).

Tab. 4. Concentrations of phosphorus (P), calcium (Ca) and potassium (K) in leaves and roots of eggplant (*Solanum melongena* L.) and sunflower (*Helianthus annuus* L.) grown without arbuscular mycorrhizal fungus (–AMF) or inoculated with *Rhizophagus intraradices* (+AMF) under control or Mn toxicity conditions (800 µM as $MnCl_2$) for four weeks. Data are means (n = 4) ± SD. Differences among data of each species indicated by different letters are statistically significant ($P < 0.05$).

			Leaves		
Species	Treatments		P	Ca	K
Eggplant	Control	–AMF	0.41±0.07^{c}	17.8±0.20^{c}	36.1±0.7^{a}
		+AMF	1.55±0.07^{b}	18.2±0.25^{c}	22.1±0.6^{bc}
	Mn toxicity	–AMF	1.11±0.11^{b}	21.1±0.70^{b}	20.7±2.6^{c}
		+AMF	3.18±0.55^{a}	26.9±0.72^{a}	24.6±1.5^{b}
Sunflower	Control	–AMF	1.52±0.20^{b}	66.8±6.8^{b}	37.3±2.1^{d}
		+AMF	1.84±0.04^{b}	75.8±9.6^{b}	46.6±2.7^{c}
	Mn toxicity	–AMF	1.77±0.21^{b}	97.8±8.7^{a}	49.5±3.4^{b}
		+AMF	3.16±0.55^{a}	113.6±6.4^{a}	78.2±3.5^{a}
			Roots		
			P	Ca	K
Eggplant	Control	–AMF	4.82±0.72^{c}	13.6±0.43^{b}	10.9±0.6^{c}
		+AMF	6.86±0.76^{b}	10.9±0.85^{c}	11.3±1.3^{c}
	Mn toxicity	–AMF	10.0±0.64^{a}	18.7±0.44^{a}	19.2±2.1^{a}
		+AMF	9.66±1.06^{a}	11.1±0.24^{c}	14.1±0.8^{b}
Sunflower	Control	–AMF	1.36±0.12^{b}	51.7±3.5^{ab}	27.9±2.8^{a}
		+AMF	1.63±0.35^{ab}	57.5±6.7^{a}	26.0±3.6^{a}
	Mn toxicity	–AMF	1.64±0.29^{ab}	30.9±1.5^{c}	27.3±2.8^{a}
		+AMF	2.15±0.28^{a}	42.6±5.8^{b}	30.9±2.6^{a}

K concentration in leaves decreased in response to AMF inoculation in eggplant but increased in sunflower under control conditions, while root K concentration was not affected in either species (Tab. 4). Mn toxicity reduced leaf K concentration in eggplant but increased it in sunflower. In the roots, exposure to excess Mn increased K concentration in eggplant but did not affect it in sunflower. AMF inoculation of Mn-stressed plants increased leaf K concentration, particularly in sunflower, but decreased root K concentration in eggplant and had no effect in sunflower (Tab. 4).

Discussion

Manganese toxicity is a frequent issue in plant production. However, due to substantial differences among plant species in their susceptibility to excess Mn and variation in the expression of Mn toxicity (Alejandro et al. 2020), studies on unexamined species may expand our understanding of mechanisms underlying plant tolerance to excessive Mn. Although numerous studies have addressed the alleviation of heavy metal toxicity through plant association with AMF, most focus on a limited number of heavy metals, primarily Cd (Dhalaria et al. 2020, Riaz et al. 2021), while information on the mechanisms through which AMF association alleviates Mn toxicity in plants remains limited.

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The difference between eggplant and sunflower in mycorrhizal colonisation and response to excess Mn

Sunflower plants exhibited higher colonisation than eggplants under both control and Mn toxicity conditions (On-line Suppl. Tab. 1). Interspecific variation in root colonisation with the same AMF species may arise from differences in host plant preference, the type and concentration of root signals and exudates, and resource allocation strategies between the fungus and plant (Klironomos 2003, Smith and Read 2008). In our study, limited carbohydrate supply to the fungal partner may partly explain the lower root colonisation in eggplant compared with sunflower. While both soluble sugars and starch levels increased in sunflower roots upon mycorrhizal colonisation, soluble sugar content in +AMF eggplant roots was lower than in –AMF counterparts. Positive correlations between carbohydrate availability in roots and AMF formation have been reported (Muthukumar and Udaiyan 2000, Salmeron-Santiago et al. 2023). High carbohydrate concentrations in roots are associated with higher colonisation rates, even at low carbon influx (Muthukumar and Udaiyan 2000), suggesting that AMF acts as a strong carbon sink. Since root starch content in eggplant was higher in +AMF plants than in –AMF ones, this may indicate that the plants could not efficiently mobilise stored carbon to support the fungal partner. Additionally, lipids are crucial carbon sources delivered to the fungal symbiont (Feng et al. 2020), which warrants further investigation.

Exposure to excess Mn decreased root colonisation in both species, as expected. Heavy metals inhibit AMF growth and development due to direct toxicity to fungal cells and disruption of fungal metabolism, resulting in decreased root colonisation (Pawlowska and Charvat 2004, Hildebrandt et al. 2007, Robinson et al. 2021). Some AMF species exhibit adaptive mechanisms, including metal sequestration within fungal cells, production of chelating compounds, and modification of fungal cell walls to reduce metal uptake. These mechanisms vary among AMF species and depend on the heavy metal involved (Gonzalez-Guerrero et al. 2008, Janoušková and Pavlíková 2010). Metal-induced reductions in plant growth and metabolism may also negatively impact AMF association by decreasing the release of root signalling molecules for symbiosis initiation and limiting carbon supply to the fungal partner (Zhang et al. 2020). This latter mechanism is supported in our study by the observed lower carbohydrate levels in roots under excessive Mn supply in both species (Tab. 3).

The difference in the expression of leaf symptoms between the two species

Leaf symptoms differed between the two species. Eggplant leaves exhibited dark-brown spots and light-brown speckles that lacked any connection to stellate trichomes (Fig. 2), whereas sunflower leaves showed smaller black spots localised at the leaf trichomes (Fig. 3). The dark coloration of Mn toxicity symptoms has been attributed either to the accumulation of oxidised Mn (Horiguchi 1987) or to the colocalisation of oxidised Mn with oxidised phenolics, with the colour mainly derived from phenolics (Wissemeier and Horst 1992). As hot ethanol treatment solubilises and removes phenolics (Haminiuk et al. 2014), the persistence of spots and speckles in eggplant leaves (Fig. 2) and of small black spots at the trichome bases in sunflower (Fig. 3) suggests that these symptoms are associated with the deposition of oxidised Mn rather than oxidised phenolics.

Previous studies using synchrotron-based micro-X-ray fluorescence (μ-XRF) analysis has shown that darkened, non-glandular trichomes in sunflower leaves under Mn toxicity are associated with Mn accumulation in the apoplast of basal trichome cells, particularly in higher oxidation states (Blamey et al. 2018). Our visual observations (Fig. 3) support these findings and corroborate this distinct localisation pattern in the basal trichome cells. In contrast, species lacking

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trichome-based Mn accumulation, such as soybean, show Mn deposition in the apoplast of epidermal cells (Blamey et al. 2018). In our study, the severe lesions observed in leaf cross sections corresponding to dark-brown spots in eggplant (Fig. 2c, d) likely result from Mn deposition in both the apoplast and symplast.

The distribution of symptoms within the leaf blades also differed between the two species. Given the association between leaf spots and speckles and sites of Mn accumulation (Blamey et al. 2018), we speculate that the greater occurrence of Mn-related symptoms in the midribs and certain second-order veins of eggplant (Fig. 2a, b), in contrast to the even distribution of black spots in sunflower (Fig. 3), reflects relatively low Mn mobility within the leaf tissue of the former species. This may partly be explained by the constitutively higher POD and PPO activities in eggplant—approximately two orders of magnitude higher than in sunflower (Fig. 7). These enzymes can oxidise Mn(II) to insoluble Mn(IV) (Fecht-Christoffers et al. 2006), leading to Mn deposition in xylem vessels or in mesophyll cells near the leaf base (Fig. 2a, b).

Efficient compartmentalisation of heavy metals in outer leaf layers, such as the epidermis and trichomes, minimises their impact on sensitive tissues, including photosynthetic cells, and has been reported in some species. Hyperaccumulators, such as *Alyssum murale* (Tappero et al. 2007, McNear and Kupper 2014), *Arabidopsis halleri* (Küpper et al. 2000), and *Brassica juncea* (Salt et al. 1995), store excessive Mn and Co in trichomes rather than other epidermal cells. Trichomes also contribute to Cd detoxification in non-accumulators such as *Arabidopsis thaliana* (Gao et al. 2021) and *Nicotiana tabacum* (Choi et al. 2001). Sunflower trichomes also store foliar-applied Zn, contributing to leaf Zn homeostasis (Li et al. 2021).

Leaf pigments and photochemistry as affected by AMF colonisation and Mn toxicity

Unlike eggplant, sunflower did not exhibit visible chlorosis (Figs. 2, 3), consistent with leaf Chl concentration data (Tab. 1), which showed a smaller reduction in sunflower (19%) than in eggplant (61%) (Tab. 1, On-line Suppl. Tab. 1). Mn toxicity also reduced leaf Car content similarly in both species. Loss of pigments under Mn toxicity has been linked to the accumulation of ROS and disruption of thylakoid membranes (Millaleo et al. 2010, Fernando and Lynch 2015). Leaf Chl and Car contents increased consistently with AMF inoculation in both species. In eggplant, increased Car in +AMF plants corresponded with higher qN (Tab. 2), an indicator of Car-mediated heat dissipation in chloroplasts (Maxwell and Johnson 2000).

Chlorophyll fluorescence parameters are sensitive indicators of PSII function and are widely used to assess photosynthetic light reactions under stress (Kalaji et al. 2016). Mn toxicity did not significantly affect these parameters in sunflower but decreased them in eggplant, consistent with species-specific differences in Chl reduction, toxicity symptom severity, and biomass under excess Mn. Sequestration of Mn at the base of leaf trichomes (Blamey et al. 2018)—evidenced by our visual observations (Fig. 3)—likely protected mesophyll cells, explaining differences in pigment loss and photosynthetic performance between species.

Under control conditions without Mn toxicity, AMF improved photochemical parameters in sunflower (Tab. 2), potentially through enhanced stomatal conductance, higher CO_2 assimilation, and increased expression and activity of Calvin cycle enzymes, as reported in other species (Chen et al. 2017, Xu et al. 2024). These coordinated changes protect PSII from over-excitation, improving light reactions (Tab. 2) and carbohydrate content (Tab. 3) even in the absence of external stress.

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The role of the antioxidant defence system in AMF-mediated responses and Mn toxicity tolerance

Excess heavy metals induce ROS accumulation, particularly in leaves, through single-electron transfer reactions with oxygen, redox imbalances due to reduced carbon reactions, and excess excitation energy in plastids (Rodrigo-Moreno et al. 2013, Shahid et al. 2014). Antioxidant enzymes play a crucial role in mitigating heavy metal toxicity. In eggplant, APX activity increased significantly under Mn toxicity, while CAT activity remained unchanged, and SOD activity decreased (Fig. 5). In contrast, all three antioxidant enzymes increased in sunflower under toxic Mn exposure. These observations suggest that, besides lack of a trichome-based Mn accumulation, a lower capacity for antioxidant induction may contribute to eggplant's higher susceptibility.

AMF can activate plant antioxidant defences under both stressed and non-stressed conditions (Hajiboland et al. 2010, Zhu et al. 2022, Ahammed and Hajiboland 2024). In the present study, however, a pronounced increase in antioxidant enzyme activities was not detected following mycorrhisation in either species, and in some cases their activities even declined in eggplant. Nevertheless, this pattern was not reflected in leaf H_2O_2 levels (Fig. 4). In fact, H_2O_2 content was consistently lower in +AMF plants than in –AMF plants in both species. This indicates that upregulation of antioxidant defences was not necessary, at least during the experimental period and under the applied Mn concentrations.

The AMF-mediated reduction in H_2O_2 levels was accompanied by a lower MDA concentration in eggplant, indicating reduced lipid peroxidation and, consequently, lower oxidative stress. In sunflower, however, MDA accumulated to higher levels following AMF inoculation under both control and Mn-toxic conditions (Fig. 4). This increase occurred despite a significant enhancement in biomass in +AMF plants. The lack of correspondence between biomass response and MDA levels suggests that other key factors, such as improved photosynthetic light reactions, higher leaf Chl and Car contents, and enhanced macronutrient status, played a more dominant role in shaping the overall response of sunflower to AMF.

Biosynthesis and accumulation of phenolics in response to Mn toxicity and mycorrhizal colonisation

The phenylpropanoid pathway, responsible for phenolic biosynthesis and accumulation, is commonly upregulated under heavy metal exposure (Michalak 2006). In this study, toxic Mn concentrations rarely influenced PAL and PPO activities in either species (Fig. 7). Leaf total phenolics responded differently: they decreased in eggplant but increased in sunflower under excess Mn (Fig. 6, On-line Suppl. Tab. 1). Similar trends were observed for anthocyanins (Tab. 1). The elevated phenolic and anthocyanin contents in sunflower may substantially contribute to its higher Mn tolerance by providing antioxidant protection and directly chelating and sequestering Mn^{2+} (Hider et al. 2001, Tolrà et al. 2009). Anthocyanins, in particular, have been implicated in metal homeostasis and are known to form complexes with Mn^{2+} as well as other metal ions (Landi 2015, Fedenko et al. 2022).

Studies on various plant species have reported enhanced accumulation of phenolic compounds following AMF inoculation (Kaur and Suseela 2020, Santos et al. 2020, Muniz et al. 2021, Amani Machiani et al. 2022). In the present study, an AMF-mediated increase in total free phenolics was observed only in the leaves of Mn-stressed plants. Although the analysis was limited to total phenolics and did not distinguish among individual phenolic classes, this increase may represent an additional component of the AMF-associated response to Mn toxicity in both species.

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The effect of AMF on macronutrients level

The concentrations of the three major macronutrients (P, Ca and K) were affected by both AMF inoculation and Mn toxicity, with responses differing markedly between the two species (Tab. 4, On-line Suppl. Tab. 1). Mycorrhizal colonisation is known to enhance P acquisition (Begum et al. 2019, Ahammed and Hajiboland 2024), but in this study, soluble P was supplied in the nutrient solution, reducing the likelihood that AMF improved P availability through rhizosphere solubilisation. Nonetheless, AMF can enhance P uptake through improved root growth (Fig. 1e, f) and nutrient foraging by extraradical hyphae. In the absence of Mn toxicity, AMF-mediated improvement of P status was significant in both leaves and roots of eggplant, whereas in sunflower this effect was not significant in either organ (Tab. 4). This finding suggests a higher 'mycorrhizal dependency' in eggplant than in sunflower despite the lower colonisation rate observed in eggplant roots (Fig. 1a, b). Mycorrhizal dependency reflects the degree to which plant growth and nutrient acquisition rely on mycorrhizal symbiosis and varies widely among plant species (Janos 2007). Species with a lower intrinsic ability to acquire P from the soil—often due to less efficient root systems—tend to exhibit greater dependency on AMF colonisation for optimal nutrient uptake (Janos 2007). Under Mn toxicity, however, both species showed a similar response to AMF inoculation with respect to leaf P concentration (Tab. 4). This pattern suggests that improved P allocation to the shoots may play an important role in AMF-mediated alleviation of Mn toxicity in both species. Enhanced P nutrition in leaves may support metabolic processes, maintain photosynthetic activity, and contribute to improved tolerance to Mn-induced stress in both species.

Under Mn toxicity, AMF colonisation increased Ca and K concentrations in eggplant leaves, accompanied by a decrease in their concentrations in the roots (Tab. 4). This pattern suggests enhanced root to shoot translocation of these nutrients in mycorrhizal plants. In sunflower, however, AMF-induced increases—slight or significant—in leaf Ca and K concentrations occurred without corresponding decreases in their root levels. This indicates that sunflower possesses a greater capacity for both uptake and root to shoot transport of Ca and K under excess Mn. Excess Mn can impair the acquisition of Ca^{2+} and K^{+} ions through competition at uptake sites and disruption of membrane transport systems (Alejandro et al. 2020, Skórka et al. 2023). In our study, however, Mn-induced reductions in these nutrients were limited, occurring only in the root of sunflower for Ca and in the leaf of non-mycorrhizal eggplant for K (Tab. 4). In contrast, Ca, K, and P concentrations generally increased under Mn toxicity, particularly in eggplant, most likely reflecting a concentration effect associated with reduced biomass under Mn stress.

The effect of AMF on leaf and root accumulation of Mn

AMF can reduce heavy metal accumulation in plant shoots through several mechanisms. The AMF mycelium may act as a metal sink, sequestering metals in both extraradical and intraradical hyphae, and thereby limiting their transport to the shoot (Dhalaria et al. 2020). X-ray fluorescence (XRF) studies have shown Cd accumulation in fungal cell walls and in electron-dense granules, whereas the cytoplasm remains largely free of the metal (Nayuki et al. 2014). Vesicles and spores may also function as storage sites for metals such as Cd and Zn (Weiersbye et al. 1999, Joner et al. 2000). Another possible mechanism for the reduction of heavy metals in shoots following mycorrhizal colonisation is the involvement of glomalin, an insoluble glycoprotein produced and secreted by AMF, which can bind heavy metals within hyphae and spores (González-Chávez et al. 2004). In addition, secreted glomalin may immobilise heavy metals in the mycorrhizosphere (González-Chávez et al. 2004), thereby reducing metal availability to roots and, consequently, to

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leaves. It has been reported that glomalin production can be stimulated under metal stress (Malekzadeh et al. 2016, Qiu et al. 2022, Tan et al. 2023). However, most of this evidence concerns heavy metals other than Mn, and direct evidence for Mn binding to glomalin—either within roots or in the mycorrhizosphere—is still lacking.

In our study, +AMF plants showed significantly lower Mn accumulation in the leaves under excess Mn, which may be explained by Mn sequestration within fungal structures, as reported for other plant species (Garcia et al. 2020). However, because AMF-mediated reductions in Mn concentration were also observed in the roots, this may indirectly indicate Mn immobilisation in the growth substrate by AMF-derived Mn-binding compounds, possibly including glomalin. Nevertheless, this assumption requires further investigation, particularly through direct measurements of glomalin in roots and in the substrate. It should also be noted that perlite was used as the growth medium in this experiment, which differs chemically and physically from soil; therefore, this interpretation should be considered within the limitations of the present experimental conditions.

Conclusion

Biomass production and leaf symptoms indicate greater Mn tolerance in sunflower than in eggplant. This difference is likely related to the accumulation of Mn at the base of leaf trichomes, which limits its entry into the symplast of leaf cells, together with increased levels of phenolics and anthocyanins that act as Mn-chelating and protective compounds in sunflower—mechanisms that appear to be absent in eggplant. Sunflower roots exhibited higher AMF colonisation, likely due to more efficient carbon allocation supported by higher leaf Chl and photosynthetic activity in +AMF plants. Despite differences in colonisation and stress inhibition, both species benefited similarly from AMF under Mn toxicity. AMF enhanced leaf photosynthetic parameters, macronutrient contents, and the accumulation of protective molecules, while reducing H_2O_2 levels. AMF-inoculated plants showed reduced Mn concentrations in both leaves and roots, providing indirect evidence for Mn immobilisation in the mycorrhizosphere. Overall, multiple mechanisms contribute to the AMF-mediated mitigation of Mn toxicity, independent of species-specific differences.

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Author contributions

ZB carried out the experiment and performed all biochemical and mineral analyses. RH conceived the study, designed the experiment, interpreted the results, and wrote the manuscript. FN contributed to the plant analyses. NA contributed to the experimental design and revised the manuscript. All authors have read and approved the final version of the manuscript.

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