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Manganese-induced oxidative stress in two ontogenetic stages of chamomile and amelioration by nitric oxide

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ABSTRACT

Impact of manganese (Mn^{2+}) excess (100, 500 and 1000 μM over 7 days) on two ontogenetic stages (7-week-old plants and 7-day-old seedlings) of *Matricaria chamomilla* was compared. Mn excess depressed growth of seedlings (but not germination) and stimulated oxidative stress (ROS and lipid peroxidation) in both plants and seedlings. Growth inhibition could be evoked by higher Mn uptake and higher translocation factor in seedlings than in plants. Total thiols staining revealed elevation in almost all treatments. In 7-week-old plants, activity of peroxidases increased slightly and rather decreased under high Mn doses. Superoxide rather than hydrogen peroxide contributed to visualized ROS presence. Fluorescence of nitric oxide (NO) showed stimulation in plants but decrease in seedlings. Impact of exogenous nitric oxide donor (sodium nitroprusside/SNP) was therefore tested and results showed amelioration of 1000 μM Mn-induced oxidative stress in seedlings (decrease in H_2O_2 and increase in NO content while antioxidative enzyme activities were variably affected) concomitantly with depleted Mn accumulation. It is concluded that NO participates in tolerance to Mn excess but negative effects of the highest SNP dose were also observed. Extensive fluorescence microscopy is also explanatively discussed.

1. Introduction

Increasing industrial production leads to elevated release of undesirable heavy metals into the environment and represents increasing risk for human health if accumulated in crop plants. On the hand, numerous metals are essential for plant growth and productivity but may be phytotoxic at higher concentrations. Among them, manganese (Mn) is important micronutrient being e.g. component of plant photosystem II [1,2]. Its toxicity is favored in acidic soils where amount of Mn^{2+} increases and it can be easily absorbed and translocated within plant leading often to growth inhibition [3,4].

Heavy metals stimulate increase in ROS (reactive oxygen species) formation, being affected especially by the given metal and exposure concentration [5]. This phenomenon was also observed in terms of Mn excess though with substantially lower intensity considering applied Mn concentrations [6]. This oxidative stress may be controlled by various antioxidative enzymes [7,8]. Their activity in Mn-exposed plants is usually affected only slightly e.g. at the level of peroxidases [9]. Though harmful if present in excess, ROS may also modulate plant growth [10]. On the contrary to ROS, nitric oxide (NO) typically exhibits protective effect in plants through changes in relation to ROS [11]. Its involvement in Mn toxicity has only rarely been studied [12]. Surprisingly, changes to NO content under Mn excess have not yet been observed/visualized and eventual toxicity of widely used NO donor (sodium nitroprusside, SNP) is only partially known [13]. Typically, SNP ameliorates metal-induced depression of growth and toxicity in various plants [14–17].

Mn uptake was studied in numerous species and it is well known that it accumulates considerably in plant biomass [6,18]. Chamomile (*Matricaria chamomilla*) is a widely used medicinal plant cultured throughout the world. It accumulates considerable amount of some metals such as Cd and Ni in the shoot [19]. Other metals, such as Al, are only slightly accumulated [20]. It was therefore the aim of the present study to verify Mn uptake and subsequent alteration of oxidative stress-related parameters in chamomile treated by three Mn doses. Owing to various cultivation and species analyzed, comparison of Mn uptake is often complicated. We therefore cultured older plants in hydroponics and compared responses with seedlings germinated directly in solutions with identical Mn concentrations.

Fluorescence microscopy is modern technique used in physiology [21,22]. It allows visualization of target compounds using (usually) specific probes. Such visual data are suitable to see localization of target compound(s) within tissue or organs. Here we provide extensive fluorescence microscopy of selected oxidative stress-related parameters in Mn-exposed plants for the first time.

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For complexity, we also compare these responses in two ontogenetic stages of chamomile mentioned above. Eventual involvement of NO in Mn-induced toxicity was also studied in chamomile seedlings.

2. Materials and methods

2.1. Plant culture, experiments design and statistics

Twenty-one days old seedlings of *M. chamomilla* L. (tetraploid 'Lutea', Asteraceae) germinated in sand were placed to Hoagland solution [11,19,20]. Uniform plants were cultivated in dark plastic boxes with 5 L of continually-aerated solutions (25 plants per box). The experiment was performed in a growth chamber under controlled conditions: 12-h day (6.00 am–6.00 pm), the photon flux density was ~270 μmol m^{-2} s^{-1} PAR at leaf level supplied by cool white fluorescent tubes L36W/840 (Lumilux, Osram, Germany) with a 25/20 °C day/night temperature and relative humidity of ~60%. In these conditions, plants form basal leaf rosettes only (=shoots). Solutions were renewed weekly to prevent nutrient depletion. Plants, that had been cultivated hydroponically during 4 weeks (=total age 7 weeks), were used in the experiment and further cultured for 7 days in mentioned Hoagland solution enriched with Mn^{2+} concentrations 100, 500 and 1000 μM (added in the form of $MnCl_2{\cdot}4H_2O$). Including control, all variants contained 2.03 μM Mn^{2+} as micronutrient and pH was checked to be 6.0. One box containing 25 plants was used for each treatment, thus the whole experiment included 4 boxes.

Subsequent experiment was focused on Mn^{2+} uptake and toxicity in chamomile seedlings. Seeds (100) were sown directly on filter paper placed on glass balls (2 mm in diameter) within Petri dishes containing identical Mn^{2+} concentrations of $MnCl_2{\cdot}4H_2O$ as mentioned above (but prepared using deionised water only). Solutions were applied below the surface of filter paper and were not applied directly on filter paper to ensure than Mn^{2+} can freely diffuse through filter paper toward seedlings. Seeds germinated within 48 h and they were harvested 5 days later, thus the whole exposure time was 7 days. Final Mn concentration of solutions was measured to verify that Mn was available through filter paper and selected parameters were monitored. Fluorescence microscopy was also done as mentioned below. Another experiment with seedlings was focused on eventual amelioration of toxicity induced by 1000 μM Mn^{2+} using exogenous nitric oxide supplied as sodium nitroprusside (SNP) in the final concentrations of 100, 500 and 1000 μM. Exposure was realized using mentioned Petri dishes and seeds were again sown on filter paper and experimental solutions were prepared using deionised water only. At least three dishes were used for each treatment.

For fresh mass-requiring parameters, individual plants were powdered using liquid N_2 and fresh material was extracted as described below. Dry samples (dried at 75 °C to constant weight) were analyzed for mineral nutrients and/or Mn amount.

Two independent repetitions of the whole experiment were performed in order to check reproducibility. Data were evaluated using ANOVA followed by a Tukey's test (MINITAB Release 11, Minitab Inc.; State College, Pennsylvania) at $P < 0.05$. Number of replications (n) in tables/figures denotes individual plants measured for each parameter among 7-week-old plants while seedlings within one Petri dish were pooled prior to determination of biomass production, Mn content and enzymatic assays as mentioned in respective tables (then $n = 3$ means three individual dishes).

2.2. Quantification of manganese and mineral nutrients

Samples were prepared by mineralization of dry material in the mixture of concentrated HNO_3 and water (5 + 5 ml) using microwave decomposition (Ethos Sel Microwave Extraction Labstation, Milestone Inc.) at 200 °C over 1 h. Resulting clear solution was quantitatively placed to glass flasks and diluted to a final volume of 20 ml. Chamomile seedlings (owing to very low biomass) were mineralized in lower amount of solution and diluted to a final volume of 3 ml. All measurements were carried out using an atomic absorption spectrometer AA30 (Varian Ltd.; Mulgrave, Australia) and the air-acetylene flame. Samples for quantification of "intra-root" Mn were washed with 5 mM Na_2-EDTA before drying. Measurements of mineral nutrients were done as described previously [23] and Mn was quantified at λ_{max} = 279.5 nm. LOD of Mn was 2 μg/l.

2.3. Fluorescence microscopy

In 7-week-old plants, freshly prepared hand-made cross sections were stained. Primary roots were excised ca. 5 cm from the leaf rosettes' base (ca. 3 cm below the surface of cultivation solutions) in the zone of lateral roots formation. In the shoots, adult leaf's petioles of similar age from 3 individual plants were stained. Among seedlings, whole fresh roots or cotyledons were stained. We observed root apex and root in the zone of differentiation, where root hairs appear. ROS and RNS/NO were stained using CellROX® Deep Red Reagent ($644_{ex}/665_{em}$, Life Technologies Corporation) and 2,3-diaminonaphthalene (Sigma–Aldrich) forming highly fluorescent 1H-naphthotriazole product ($365_{ex}/415_{em}$) in accordance with manufacturer's instructions. Stock solution of CellROX® Deep Red Reagent in DMSO was diluted by PBS buffer (0.05 M, pH 6.8) to final concentration of 5 μM and samples were stained for 60 min at 37 °C. Stock solution of 2,3-diaminonaphthalene in 0.62 M HCl was used diluted by PBS buffer (0.05 M, pH 6.8) to the final concentration of 250 μM. After incubation, samples were washed three times by PBS buffer and observed. Amplex® UltraRed ($568_{ex}/681_{em}$ nm, Life Technologies Corporation) was used to visualize hydrogen peroxide in accordance with manufacturer's instructions. Briefly, plant tissues were incubated in a working solution that consisted of 50 μl of 10 mM Amplex® UltraRed stock solution in DMSO, 100 μl of horseradish peroxidase (10 U/ml in 0.05 M PBS buffer, pH 6.0, Sigma–Aldrich, USA) and 4.85 ml of reaction buffer (0.05 M PBS, pH 6.0). Plant tissues were incubated for 30 min at room temperature and dark to avoid accelerated oxidation. After incubation, plant tissues were carefully washed three times with PBS buffer (0.05 M, pH 6.0) and observed. Lipid peroxidation was visualized by BODIPY® 581/591 C11 lipid peroxidation sensor ($581_{ex}/591_{em}$ nm, Life Technologies, USA) where oxidation of the polyunsaturated butadienyl portion of the dye results in a shift of the fluorescence emission peak from 590 to 510 nm according to manufacturer's instructions. Stock solution in ethanol was diluted by PBS buffer (0.1 M, pH 7.4) to a final concentration of 4 μM. Samples were stained for 30 min at RT and darkness to avoid accelerated oxidation. Thereafter, they were washed three times and observed. The fluorescence of BODIPY® 581/591 C11 was acquired simultaneously using dual excitation (485 and 581 nm) and detection (510 and 591 nm). Final images represent merges of 510 and 591 nm emissions. All staining were carried out in the dark to avoid possible light-accelerated oxidation. Total thiols were stained with monochlorobimane (Sigma–Aldrich, USA). Stock solution of monochlorobimane ($394_{ex}/490_{em}$) in methanol (11 mM) was used to prepare working solution. Working solution was prepared immediately prior to use by diluting stock solution with PBS (0.05 M, pH 7.2) to a final concentration of 50 μM. Samples were stained for 20 min in the dark at room temperature, washed three times to remove excess of staining solution and observed [21]. Fluorescence microscopy was done with Axioscop 40 microscope (Carl Zeiss, Germany) equipped with appropriate set of excitation/emission filters.