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Smelling the metal: Volatile organic compound emission under Zn excess in the mint *Tetradenia riparia*

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ABSTRACT

This work investigated the effect of Zn excess on growth, metal accumulation and photosynthetic changes in *Tetradenia riparia,* in relation to possible variations in the composition of the plant volatilome. Experiments were carried out in hydroponics exposing plants to a range of Zn concentrations. Zinc excess negatively affected plant growth in a dose-dependent manner. The metal was accumulated proportionally to its concentration in the medium and preferentially allocated to roots. All the photosynthetic parameters and the concentration of some photosynthetic pigments were negatively affected by Zn, whereas the level of leaf total soluble sugars remained unchanged.

Twenty-three different VOCs were identified in the plant volatilome. Each compound was emitted at a different level and intensity of emission was manifold increased by the presence of Zn in the growth medium. The Zn-induced compounds could represent both an adaptive response (f.i. methanol, acetylene, C_6-aldehydes, isoprene, terpenes) and a damage by-product (f.i. propanal, acetaldehyde, alkyl fragments) of the metal presence in the culture medium. Given that the Zn-mediated induction of those VOCs, considered protective, occurred even under a Zn-limited photosynthetic capacity, our work supports the hypothesis of an active role of such molecules in an adaptive plant response to trace metal stress.

1. Introduction

Trace metallic elements are naturally present in the environment, but human activities, such as mining, industry and agriculture, have led to concern for the raising in their concentration in the environmental matrices all over the world [1]. Such elements, despite some of them are micronutrients for the living organisms, are potentially toxic, in relation to the concentrations of their bioavailable form and the sensitivity of the exposed organisms [2]. Actually, while animals can move to avoid trace metal-contaminated areas, plants can only face the occurrence of element excess in their environment. Hence, in presence of trace metal stress, plants display reduced biomass, leaf chlorosis, inhibited root growth and morphological alterations and eventually die when the exposures is excessive [3].

Among the essential trace metallic elements, Zn is a cofactor of many proteins, where it can play a structural and a catalytic role, and it is the only metal represented in all known enzyme classes [4,5]. In crops, Zn is required, at a leaf concentration around 15–20 μg g^{-1} dry weight [6]. Zinc toxicity generally occurs when leaf concentrations reach 100–400 μg g^{-1} of dry mass [7]. Actually, Zn is considered as one of the most widespread trace metallic elements polluting the environment [8], due to atmospheric deposition from industries, use of Zn-polluted water, sediments and wastes and Zn fertilizers [9]. In plants, common Zn toxicity symptoms include reduced water content and stunted growth [10], changes in root growth and morphology, severe nutrient imbalances, leaf chlorosis [4,6,10,11], and decreased stomatal conductance and photosynthetic efficiency [12]. Photosynthesis impairment appears to be mainly linked to the direct inhibition of photosystems caused by high concentrations of Zn. In fact, this metal can cause the displacement of Mn at the water splitting site in photosystem II [13,14]. Furthermore, excessive Zn supply was found to greatly reduce ATP synthesis and activity in chloroplasts [15]. In addition, an excess of this metal leads to an increase in reactive oxygen species (ROS) that can induce oxidative stress thus damaging major biomolecules including lipid, protein, and nucleic acid [see for example 16 and 17]. Among the still unexplored aspects of Zn effects on plants, there is

Abbreviations: A_N, Net photosynthetic rate; g_s, Stomatal conductance; ETR, Electron transport rate; ΦPSII, Actual photosystem II efficiency; Chl *a*, Chlorophyll *a*; Chl *b*, Chlorophyll *b*

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surely the plant response to Zn excess in terms of emission of volatile organic compounds (VOCs), even though the role of such molecules in plant stress biology is receiving increasing attention.

Volatile organic compounds produced by vegetation include a diverse set of chemical molecules mainly represented by isoprene, monoterpenes and methanol [18]. In any case, the variety of plant-emitted VOCs depends on the plant species, the distinctive parts of the plants, or the growth conditions [19]. Plants can release larger quantities of such molecules, due to an increased investment of carbon into stress-inducible VOCs, when they are attacked by pathogens or herbivores, or have to face abiotic stresses, such as UV, ozone, drought, eutrophication and warming, and, more generally, oxidative stresses [20–23]. Actually, many VOCs of the family of terpenes, such as isoprene and monoterpenes, are antioxidants and can protect cells from oxidative stress [20,24].

Despite the well-known involvement of several VOCs in plant stress protection and communication, the response of volatile emission, either quantitative or qualitative, to trace metallic elements in excess has been little investigated. To the best of our knowledge, there is only one report of a significant metal-induced emission of VOCs [25] in the case of Cu and one in the case of Zn [26].

The mint *Tetradenia riparia* (Hochst.) Codd. (Lamiaceae), commonly known as “false myrrh”, is a widespread aromatic shrub occurring throughout eastern tropical Africa [27]. *Tetradenia riparia* can occupy a wide range of ecologically contrasting habitats, but it is often found on hillsides and river banks [28]. The Lamiaceae family is abundant in aromatic species, used as culinary herbs, fragrant scents and folk medicines. In particular, *T. riparia* is a well-known herbal medicine and has been traditionally and widely used in the treatment of various illnesses including coughs, dropsy, fever, malaria [28,29] and, recently, also leishmaniasis [30]. The essential oil of *T. riparia* has been shown to be a complex mixture of terpenoids, including monoterpenes, sesquiterpenes, and diterpenes [31]. Hence, *T. riparia* can represent a helpful model system to investigate the relationship between trace metal stress and volatile emission. In addition, the impact of trace metal pollution on VOC composition claims to be studied considering the increasing levels of Zn all over the world, South Africa included [32], where this mint is traditionally cultivated and used. In the present study, we examine the effect of Zn on growth, metal accumulation and photosynthetic changes in *Tetradenia riparia*, and tested if the exposure to Zn can induce a response in terms of qualitative and/or quantitative changes in the composition of the plant volatilome. To our knowledge, this work is the first complete analysis of plant emitted VOCs in presence of increasing concentration of a trace metallic element.

2. Materials and methods

2.1. Plant material

Plant material was cultivated as in Bazihizina et al. [33]. *Tetradenia riparia* plants grown for 1 year in a naturally lit glasshouse in standard potting mix were used to obtain cuttings (3 internodes). After 3 weeks in aerated water in the same glasshouse, rooted cuttings were transferred to 5 L plastic pots (6 plants per pot) containing an aerated half-strength Hoagland’s nutrient solution [34] composed of 3 mmol L^{-1} KNO_3, 2 mmol L^{-1} $Ca(NO_3)_2$, 1 mmol L^{-1} $NH_4H_2PO_4$, 0.50 mmol L^{-1} $MgSO_4$, 20 μmol L^{-1} Fe(Na)-EDTA, 1 μmol L^{-1} KCl, 25 μmol L^{-1} H_3BO_3, 2 μmol L^{-1} $MnSO_4$, 2 μmol L^{-1} $ZnSO_4$, 0.1 μmol L^{-1} $CuSO_4$, and 0.1 μmol L^{-1} $(NH_4)_6Mo_7O_{24}$ in milliQ-water (Millipore, Billerica, MA, USA) buffered with 2 mmol L^{-1} 2- morpholinoethanesulfonic acid (MES), pH 5.5 adjusted with KOH. The solutions were renewed weekly and two months after transferring the plants to nutrient solutions plants were selected for shoot and root uniformity and $ZnSO_4$ was added to the cultures to obtain the required final Zn concentrations (control treatment and 0.1, 0.5 and 1 mmol L^{-1}). Plants (12 plants per treatment) were grown in glasshouse for 4 weeks from the start of the treatment (average temperature 35/25 °C day/night, 15/9 h day/night during the treatment period).

2.2. Measurement of leaf gas-exchange parameters

Leaf gas-exchange parameters were measured using the open gas-exchange system Li-6400 XT (Li-Cor, Lincoln, NE, USA) with an integrated fluorescence chamber head (Li-6400-40; Li-Cor). Leaf gas-exchange measurements were taken on all plants from each treatment at the following different time points: before the start of the treatment (Time 0) and after 1, 7, 14 and 28 days, following the method described in Bazihizina et al. [33]. Briefly, the photosynthetic parameters were determined on the youngest fully expanded leaves with reference CO_2 of 400 μmol mol^{-1}, ambient relative humidity (40–50%), flow rate of 400 μmol s^{-1}, chamber temperature of 25 °C and photosynthetically active radiation of 300 μmol m^{-2} s^{-1}. The level of minimal fluorescence in the dark-adapted state (F_0) was measured using a modulated pulse, and the level of maximal fluorescence in this state (F_m) was measured after applying a saturating actinic light pulse of 7000 μmol m^{-2} s^{-1}. The values of the variable fluorescence F_v was calculated as F_m-F_0 and ΦPSII was calculated as F_v/F_m [33].

2.3. Plant harvest

At the beginning of the experiment, dry biomass of 12 plants was recorded to subsequently calculate the difference between dry biomass at the end of the treatment and at its onset. After 4 weeks of treatment, randomly selected young fully expanded leaves (one for each of the 12 plants per treatment) were collected, freeze-dried and stored at −80 °C for the analyses of leaf pigments and total soluble sugars.

Plant roots were carefully washed with a solution of 10 mmol L^{-1} $Pb(NO_3)_2$ for 30 min to desorb metals adhering to the root cell wall, as in Arnetoli et al. [35]. Each plant was then washed with milliQ-water, separated into roots, stems and leaves. All plant material was then oven-dried at 60 °C for 1 week for dry mass determination.

2.4. Measurement of zinc concentration

Zinc concentrations were determined in roots, stems and leaves by acid digestion, as described in Pignattelli et al. [36]. Oven-dried plant material was ground using an electric grinder and aliquots (about 100 mg) of each sample were mineralized in a mixture of HNO_3: $HClO_4$ (5:2 v/v) in 25 mL beakers on a heating plate (120–200° C), after which the volume was adjusted to 10 mL with milliQ-water. Zn concentration was determined in the digests by an atomic absorption spectrophotometer (AAnalyst 200, Perkin Elmer).

2.5. Leaf pigment analyses

Total chlorophyll and carotenoid concentrations were determined by reading the absorbance at 665, 652, and 470 nm of extracts obtained from randomly selected youngest fully expanded leaves, as in Rich et al. [37]. Frozen-dried leaf samples were ground in liquid nitrogen, then 0.02 g were weighed and incubated with 1.25 mL of methanol 100% for 30 min at 4 °C in the dark. Samples were then centrifuged at 9300 *g* for 10 min at 4 °C and the super-natants were used for absorbance determination by means of a Tecan Infinite 200 Spectrophotometer (Männedorf, Switzerland). Chlorophyll and carotenoid concentrations were calculated according to the equations from Wellburn [38].

2.6. Measurement of total soluble sugars

According to Yemm and Willis [39], frozen-dried leaf samples (aliquots of about 20 mg) were incubated with 80% ethanol in a 80 °C water bath for 20 min and centrifuged at 15000 rpm for 10 min. This step was repeated twice, to ensure the extraction of all soluble sugars.