RESEARCH PAPER

Effect of zinc imbalance and salicylic acid co-supply on Arabidopsis response to fungal pathogens with different lifestyles

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INTRODUCTION
Several transition metals, including zinc (Zn), are essential micronutrients for living organisms, including higher plants (Mousavi 2011). In plants, Zn is a component of structural proteins and enzymes; in particular, it is the only metal associated with all enzyme classes, mainly anhydrases, dehydrogenases, oxidases and peroxidases, and plays a crucial role in their synthesis and activity (Coleman 1998; Kraemer & Clemens 2005; Mousavi 2011; Cabot et al. 2019). According to the Arabidopsis Information Resource (TAIR) database, in A. thaliana Zn is a component of over 1200 proteins (Kraemer & Clemens 2005). Cell multiplication, auxin synthesis and balance, synthesis of nucleic acids, ribosome formation, uptake of micro- and macro-nutrients, water absorption and responses to several stress factors are among the functions in which Zn is involved in plants (Kraemer & Clemens 2005; Duffy 2007; Cabot et al. 2019). A positive correlation has been reported between Zn level in plants and in the soil (Wong et al. 2019), as well as both positive and negative interactions between Zn and other macro- and micro-nutrients (Mousavi 2011).

Given its importance, imbalances in Zn nutrition, both deficit and excess, can affect plant growth and physiology and alter plant response to biotic and abiotic stresses (Alloway 2008). Zinc deficiency is among the main plant nutrition deficiencies worldwide (Duffy 2007). Symptoms of Zn deficiency appear first on the youngest leaves as discoloration between veins in the form of bleaching, purpling or yellowing, depending on plant species; also, leaf size and internode length are negatively affected (Duffy 2007; Mousavi 2011). Leaf growth is also compromised by excess Zn, which at high concentrations has toxic effects connected to an increase in free radicals and a decrease in activity of the photosynthetic apparatus and ATP synthesis (Mousavi 2011).

Considering biotic stresses, the effect of Zn level on plant response can vary according to the host–pathogen interaction (Duffy 2007; Cabot et al. 2019). Thus, with respect to biotrophic fungal pathogens, inadequate Zn nutrition increased susceptibility of pecan trees to Mycosphaerella dendroides (Schwein.) Demaree & Cole and Cercospora fusca F.V. Rand (Moznette et al. 1940), as well as susceptibility of rubber trees to Oidium spp. (Bolle-Jones & Hilton 1956). In contrast, Zn-deficient soybean plants showed less susceptibility to Phakopsora pachyrhizi Syd. & P. Syd. (Helfenstein et al. 2015). An increase in susceptibility to the biotrophic Erysiphe graminis DC.

ABSTRACT
• In higher plants, Zn nutritional imbalance can affect growth, physiology and response to stress, with effect variable depending on host–pathogen interaction. Mechanisms through which Zn operates are not yet well known. The hormone salicylic acid (SA) can affect plant ion uptake, transport and defence responses. Thus, in this study the impact of Zn imbalance and SA co-supply on severity of infection with the necrotrophic fungal pathogen B. cinerea or the biotroph G. cichoracearum was assessed in A. thaliana Col-0.
• Spectrophotometric assays for pigments and malondialdehyde (MDA) content as a marker of lipid peroxidation, plant defensin 1.2 gene expression by semi-quantitative PCR, callose visualization by fluorescence microscopy and diseases evaluation by macro- and microscopic observations were carried out.
• Zinc plant concentration varied with the supplied dose. In comparison with the control, Zn-deficient or Zn-excess led to reduced chlorophyll content and PDF 1.2 transcripts induction. In Zn-deficient plants, where MDA increased, also the susceptibility to B. cinerea increased, whereas MDA decreased in G. cichoracearum. Zinc excess increased susceptibility to both pathogens. Co-administration of SA positively affected MDA level, callose deposition, PDF 1.2 transcripts and plant response to the two pathogens.
• The increased susceptibility to B. cinerea in both Zn-deficient and Zn-excess plants could be related to lack of induction of PDF 1.2 transcripts; oxidative stress could explain higher susceptibility to the necrotroph and lower susceptibility to the biotroph in Zn-deficient plants. This research shows that an appropriate evaluation of Zn supply according to the prevalent stress factor is desirable for plants.
was observed in Zn-supplied wheat cultivars (Bolle-Jones & Hilton 1996).

Under Zn stress, application of the phytohormone salicylic acid (SA) may improve plant growth and alleviate damage due to Zn toxicity (Es-sibhi et al. 2020). Exogenous SA has also been reported to play an important role in ion uptake and transport (Shi & Zhu 2008; Sharma et al. 2020) and in nutrient fortification (Smolen et al. 2016). Moreover, SA has positive effects on the response to both biotic and abiotic stresses, including metal toxicity (Spletzer & Enyedi 1999; Mandal et al. 2009; Gondor et al. 2016).

In the host–fungus interaction, Zn level affects not only the plant but also the attacker. Indeed, several studies have shown that Zn is involved in phytopathogenic fungal growth, sexual morphogenesis, sporulation, spore germination and synthesis of toxins and mycotoxins (Duffy 2007).

Zinc level in plants is also important for human health, vegetables being among Zn sources in the human diet. However, some vegetables, such as cereals and legumes, although rich in Zn, are also rich in phytate, which inhibits Zn absorption in the intestinal lumen (Brown et al. 2001).

In this research, we investigated the effect of variations in Zn dose, supplied as zinc sulphate, at physiological dose, excess or absent (deficiency), on Arabidopsis thaliana Col-0 physiological and morphological parameters (pigment level, lipid peroxidation, callose deposition) and on the severity of infections with two pathogens with different lifestyles: the necrotroph Botrytis cinerea Pers. ex Fr. and the biotroph Golovinomyces cichoracearum (DC.) V.P. Heluta. In A. thaliana, the infection process of both pathogens begins with conidial germination, through phases of penetration and colonization and end with sporulation, consisting in the formation of a new inoculum. Both pathogens penetrate the intact cuticle of the host by formation of an appressorium and penetration peg (Williamson et al. 2007; Micali et al. 2008). The biotrophic colonization of G. cichoracearum proceeds with the formation of haustoria, specialized organs through which the fungus assimilates nutrients from living cells of the host, with which it establishes a trophic relationship (Szabo & Buschnell 2001). Production of cell wall apposition in the form of papillae and encasement of haustoria in papillae extensions, both containing callose, have been observed in A. thaliana during G. cichoracearum penetration and colonization, respectively, as a plant defence response (Micali et al. 2008). Increased resistance to the pathogen by induction of callose deposition requires exogenous treatment, including SA treatment (Wang et al. 2013). The necrotroph B. cinerea kills cells during colonization through a plethora of enzymes, toxins and other compounds, such as oxalic acid, and feeds on dead plant tissues (Williamson et al. 2007; Jiang et al. 2016). Moreover, B. cinerea can trigger programmed cell death (PCD) of host cells (Williamson et al. 2007). Under controlled laboratory conditions, both pathogens complete the infection cycle on A. thaliana in approximately 4–5 days (Williamson et al. 2007; Micali et al. 2008; Jiang et al. 2016).

Since, the phytohormone SA is involved in A. thaliana resistance to both B. cinerea (Ferrari et al. 2003) and G. cichoracearum (Reuber et al. 1998) and, as reported above, to influence mineral uptake and plant response to different metal levels, we here assessed the effect of Zn and SA co-supply on the above parameters.

MATERIAL AND METHODS

Plants and treatments

Seeds of A. thaliana L. Heynh. wild-type Columbia (Col-0) were surface-sterilized in 70% (v/v) ethanol and 7% (v/v) sodium hypochlorite, rinsed in sterile distilled water then sterilized in sterile distilled water at 4 °C in the dark for 48 h. With minor modifications to Zeng et al. (2018), a sterilized woody toothpick was used for each seed sown in 0.5 ml sterilized tubes containing 150 μl 0.7% w/v water agar (Agar Bios Special LL; Biolife Italiana, Milan, Italy) and filled with 1/2 Hoagland nutrient solution. Tubes were kept in a growth chamber at 22 °C/20 °C day/night, 60–75% relative humidity and 14-h light photoperiod at a photosynthetic photon fluence rate of 120 μmol m−2 s−1 supplied by daylight lamps (Powerstar HQI-T 400 W/D daylight lamp; Osram) and fluorescent lamps (Philips TLD, the Netherlands). Two weeks after sowing, plants were moved to buckets filled with nutrient solution that was replaced every 2 weeks.

Treatments were provided to each bucket as water solution of zinc sulphate (ZnSO₄·7H₂O; Merck, Darmstadt, Germany) at 2 μM (Zn-sufficient), 25 μM (Zn-excess) or water without ZnSO₄ (Zn-deficit). The concentration 25 μM was chosen as it is reported in the literature to mimic conditions of Zn excess in non-accumulator A. thaliana (van de Mortel et al. 2006). These solutions were also applied with added salicylic acid (SA; Merck) at 0.1 mg l⁻¹, dose in the range commonly used for exogenous applications to plants. Hoagland solution and treatments were at pH of 6.0. Since we aimed to investigate the response to fungal pathogens, only 1-week treatments were used to induce Zn deficiency or excess, thus avoiding excess stress and toxicity problems.

Zinc determination in Arabidopsis leaves

Total Zn content was determined according to the US-EPA Method 3052B (USEPA 1996) in Zn-deficient, Zn-sufficient or Zn-excess plants, co-treated or not with 0.1 mg l⁻¹ SA, at 7 dpt (days post-treatment). Briefly, plant samples were oven-dried at 60 °C for 48 h and finely ground in a mixer mill (Retsch MM200; Retsch, Verder Scientific, Bergamo, Italy). For each sample, 0.3 g powder was microwaved-digested in an ETHOS One high-performance microwave digestion system (Milestone, Sorisole, Bergamo, Italy) with 8 ml ultrapure concentrated nitric acid (65% w/w) and 2 ml hydrogen peroxide (30% w/w). The heating programme for digestion was 30 min at 1000 W and 200 °C. After cooling, the digests were diluted with Milli-Water (18.2 MΩ) up to 20 ml then filtered using a 0.22-μm filter. Zinc standard solutions was prepared by diluting the corresponding stock solutions (standards 1000 mg l⁻¹ for AAS TraceCert) with HPLC-grade water. Zinc was then determined using a Shimadzu AA-6200 atomic absorption spectrophotometer (Shimadzu, Tokyo, Japan). The accuracy was validated using a recovery test (n = 3) by adding a Zn standard solution (4 mg l⁻¹) into a mixture of a Zn-enriched sample and nitric acid prior to digestion in tubes and after appropriate dilution according to the above reported US-EPA method. The experiment was independently repeated three times with three plants per treatment.
**Pigment determination**

Leaf tissue (100 mg FW) taken at 7 dpi from Zn-deficient, Zn-sufficient or Zn-excess plants, with or without 0.1 mg l⁻¹ SA, was ground in a mortar with a pestle and pigments extracted with 1.5 ml 80% acetone. The obtained samples were centrifuged at 10 000 g for 6 min and the supernatant collected for spectrophotometric assay. Pigment absorption was recorded at 663, 645 and 470 nm with a UV-Visible spectrophotometer (V-1200, Chrom Tech, Apple Valley, MN, USA). Total chlorophylls were calculated using the formula of Arnon (1949) and carotenoids as reported by Lichtenthaler & Wellburn (1985).

Each experiment was independently repeated three times, with three plants per treatment and two leaves per plant.

**Lipid peroxidation**

Lipid peroxidation was measured in A. thaliana leaves taken at 7 dpi from Zn-deficient, Zn-sufficient or Zn-excess plants, with or without 0.1 mg l⁻¹ SA, using the malondialdehyde (MDA) assay (Heath & Packer 1968). Leaf samples (200 mg FW) were homogenized with 1.5 ml 0.1% trichloroacetic acid (TCA), centrifuged at 10 000 g for 20 min and 1.0 ml 0.5% thiobisbarbituric acid (TBA) in 20% TCA added to a 0.5 ml aliquot of the supernatant. After incubation at 95 °C for 30 min, the mixture was immediately cooled on ice, centrifuged at 10 000 g for 10 min then absorbance recorded at 532 and 600 nm with the above UV-Visible spectrophotometer. MDA content was calculated using an extinction coefficient of 155 mM⁻¹ cm⁻¹ by subtracting non-specific absorption at 600 nm from absorption at 532 nm. Each experiment was independently repeated three times, with three plants per treatment and two leaves per plant.

**Pathogens, inoculations and disease evaluations**

Two fungal pathogens with different lifestyles were chosen: Botrytis cinerea, necrotrophic agent of grey mould, and Golovinomyces cichoracearum (formerly Erysiphe cichoracearum D.C.), biotrophic agent of powdery mildew. Leaf inoculations were performed using the isolates and methods reported in Ederli biotrophic agent of powdery mildew. Leaf inoculations were performed using the isolates and methods reported in Ederli et al. (2016). 20 days post-inoculation (dpi) for B. cinerea and 7 dpi for G. cichoracearum. Following Ederli et al. (2015), for B. cinerea necrotic lesion diameter was measured, while for G. cichoracearum total number of colonies, conidiophores per colony and conidia per colony were counted under a light microscope (Carl Zeiss, Jena, Germany) at 5X magnification after clearing in 96% (v/v) ethanol at 60 °C × 15 min and staining with Trypan blue solution [0.01% Trypan blue (v/v); Merck, Darmstadt, Germany] in lactic acid:phenol:glycerol:water (1:1:1:1 v/v/v/v) (Reuber et al. 1998).

Each experiment was independently repeated three times with: for B. cinerea, five plants per treatment and five leaves per plant with each leaf drop inoculated at two places, for a total of 50 lesions per treatment; for G. cichoracearum inoculation, six leaves per treatment and six randomly selected areas (2.5 mm²) per leaf, for a total of 0.15 cm² per leaf.

**Extraction of RNA and PCR analysis**

Leaf samples were collected from plants that were Zn-deficient, Zn-sufficient or Zn-excess, with or without 0.1 mg l⁻¹ SA, at 7 dpi. Samples were snap-frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted and DNase treatment performed using the PureLink™ RNA Mini Kit and PureLink™ DNase Set (Ambion, Life Technologies, Carlsbad, CA, USA), respectively, according to the manufacturer’s protocols. For each sample, total RNA was measured with an Qubit™ 3.0 Fluorometer (Thermo Fisher Scientific, Rodano, Milan, Italy) and 500 ng total RNA was reverse-transcribed using PrimeScript™ RT-PCR Kit (Takara, Saint-Germain-en-Laye, France) according to the manufacturer’s protocol.

Semiquantitative PCR was performed using the T100™ Thermal Cycler (BioRad, Foster City, CA, USA) in a total volume of 20 µl containing 30 ng DNA template, TB Green® Premix Ex Taq™ II (Takara) and 10 µM forward and reverse primers designed for the plant defensin 1.2 (PDF 1.2) (Ederli et al. 2020) were as follows: PDF 1.2 forward 5'-TTTGCGTCCTTGGACGAC-3' and reverse 5'-TAACATGGGAGCTAAGACAT-3'; and 5'-TGGTGTCTCGAACTTCCAGCAG-3'. The PCR programme for PDF 1.2 was as reported in Ederli et al. (2021) with slight modifications. Briefly, it consisted of 94 °C for 1 min 15 s, 32 cycles of 5 s at 94 °C, 20 s at 57.5 °C and 45 s at 72 °C, followed by 6 min at 72 °C. For EF 1-α, the PCR programme differed for cycles number (40) and annealing temperature (60 °C). Amplification products (190 bp for PDF 1.2 and 120 bp for EF1-α) were separated on 1.2% agarose gels in 0.5x Tris-acetate-EDTA (TAE) buffer at 100 V for 20 min, stained with ethidium bromide and visualized with a UV transilluminator. The authenticity of the PCR products were checked by two-directional sequencing using an ABI Prism 310 genetic analyser (Perkin Elmer Life and Analytical Sciences). Band intensities were determined on a scanned filter using ImageJ software (Schneider et al. 2012). Levels of transcripts are expressed as relative amounts. The experiment was independently repeated three times with three biological replicates from two leaves per plant of three individual plants per treatment.

**Callose quantification**

According to Quaglia et al. (2017), callose deposition was quantified using an epifluorescence microscope equipped with UV filters (excitation, BP 365-395; barrier, LP 420) and ImageJ software (Schneider et al. 2012) on leaves at 7 dpi from Zn-deficient, Zn-sufficient or Zn-excess plants, with or without...
0.1 mg l\(^{-1}\) SA, after clearing by immersion for 10 min in 96% ethanol at 80 °C and staining with aniline blue (Carlo Erba Reagents, Italy). The experiment was independently repeated three times, with three plants per treatment, two leaves per plant and two photographs (area 2.5 mm\(^2\)) taken at random for each leaf.

**Statistical analysis**

Data on the effects of the different Zn doses and SA co-supply on leaf total chlorophyll, carotenoids, MDA, \(PDF1.2\) gene transcripts, callose, Zn and infection severity were separately submitted to two-way (zinc dose × SA treatment) ANOVA and significant differences compared by Duncan multiple range test \((P = 0.05)\), using the Excel extension DSAASTAT (Onofri & Pannacci 2014). Details on experimental protocols are given in the figure legends.

**RESULTS**

**Effect of differences in Zn and SA supply on leaf Zn content**

With respect to the physiological Zn dose (2 \(\mu\)M), a decrease or increase in Zn concentration in the nutrient solution resulted in significant reductions or increases of Zn content in \(A.\ thaliana\) leaves at 7 dpt, respectively (Fig. 1). In particular, Zn deprivation corresponded to only a slight decrease \((-8\%)\) in Zn accumulation in leaves, while Zn excess produced a strong increase \(+(+46\%)\) in Zn leaf concentration (Fig. 1).

Co-administration with SA did not affect the Zn content in plants supplied with Zn-deficient solution, while there was a significant, but weak, increase \(+(+5\%)\) in Zn concentration in plants treated with the physiological Zn dose, and a significant reduction in Zn content \((-11\%)\) in plants with excess Zn supply (Fig. 1).

**Effect of differences in Zn and SA supply on photosynthetic pigments and lipid peroxidation**

In plants supplied with Zn-deficient or Zn-excess solution, the total chlorophyll content at 7 dpt was significantly lower \((-37\%\) approximately) than that in plants supplied with Zn solution at physiological concentration (Fig. 2A). The different Zn doses did not have marked effects on the carotenoid content (Fig. 2B). However, according to van de Mortel et al. (2006), at the time of analysis (7 dpt), no visible chlorosis was observed in these plants (data not shown). At the same time, there was a general tendency towards an increase in chlorophyll and carotenoid content in all samples as a consequence of simultaneous SA supply, even if the differences between SA-supplied or SA-deprived samples were significant only under excess Zn conditions (Fig. 2A and B).

As regards oxidative stress, in comparison with plants supplied with Zn solution at physiological concentration, the MDA content, an indicator of lipid peroxidation, increased significantly \(+(+125\%)\) in plants supplied with Zn-deficient solution, while there were no changes in plants under excess Zn conditions (Fig. 3). Added SA did not affect the MDA content in plants supplied with physiological or excess Zn, while MDA values of plants supplied with Zn-deficient solution were...
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Fig. 3. Malondialdehyde (MDA) content in leaves of Arabidopsis thaliana Col-0 supplied with water solution of zinc sulphate at 2 µM (zinc-sufficiency) or 25 µM (zinc-excess) or water solution without zinc sulphate (zinc-deficiency), and the same solutions with 0.1 mg l⁻¹ salicylic acid (SA) added 7 days post-treatment. Each experiment was independently repeated three times, with three plants per treatment. Data were submitted to two-factor (zinc dose × SA treatment) ANOVA. Each column represents mean (n = 3) ± SE. Different letters indicate significant differences (P ≤ 0.05; Duncan multiple range test).

Effect of differences in Zn and SA supply on Arabidopsis susceptibility to B. cinerea and expression of PDF 1.2

Zinc supply beyond the physiological range increased A. thaliana Col-0 susceptibility to the necrotrophic fungal pathogen B. cinerea (Fig. 4). Instead, plants supplied with Zn-deficient or Zn-excess solutions had much larger average diameter of necrotic lesions produced by the pathogen than plants supplied with Zn solution at physiological concentration (Fig. 4). These differences were particularly pronounced among plants supplied with Zn-deficient solution and Zn solution at physiological level (Fig. 4). Moreover, only in plants supplied with Zn-deficient solution the co-administration of SA significantly reduced lesion diameter (Fig. 4).

Since PDF 1.2 expression is associated with resistance to B. cinerea (Cabot et al. 2013), the transcript level of this gene was assessed. In comparison with plants supplied with Zn solution at physiological concentration, only a weak and not significant decrease in the level of PDF 1.2 transcripts was observed both in plants supplied with Zn-deficient and Zn-excess solutions (Fig. 5). The addition of SA significantly increased PDF1.2 transcript accumulation in all Zn concentrations with an increase in gene expression that was even larger the higher the Zn administration to plants (Fig. 5).

Effect of differences in Zn and SA supply on Arabidopsis susceptibility to G. cichoracearum and callose deposition

Susceptibility to G. cichoracearum increased with increasing concentrations of Zn. Indeed, as the Zn supply increased from 0 to 25 µM, the number of fungal colonies per leaf area, conidiophores per colony and conidia per colony significantly increased (Fig. 6). Addition of SA negatively affected fungal growth. Indeed, there was a marked reduction in all the considered parameters after SA co-supply (Fig. 6). Callose deposition was not affected by the different Zn concentrations but was strongly induced after SA co-supply, irrespective of Zn concentration (Fig. 7).

DISCUSSION

The impact of mineral nutrition on plant defence against biotic stresses has been investigated in recent years, with Zn as one of the most used micronutrients in these studies (Cabot et al. 2019). Zinc nutritional imbalances lead to alterations in plant physiology and changes in response to biotic stresses (Duffy 2007; Helfenstein et al. 2015; Cabot et al. 2019). The role of Zn in plant protection varies depending on the host–pathogen interaction, and both positive and negative effects on disease severity have been reported (Duffy 2007; Huber et al. 2012; Cabot et al. 2019).

Although the roles of Zn as a component of enzymatic and structural proteins and its involvement in several physiological processes are well documented (Coleman 1998; Duffy 2007; Cabot et al. 2019), mechanisms through which it operates in plant defence are not yet well known. Thus, with the aim of elucidating some of these mechanisms and their role against fungal pathogens with different lifestyles, we assessed the impact of the Zn nutritional imbalance, both deficiency and excess, on some physiological parameters and the severity of infections with the necrotroph B. cinerea or the biotroph G. cichoracearum on A. thaliana Col-0.

At 7 dpt, the leaf Zn concentration (49 mg kg⁻¹ DW) in plant supplied with Zn-sufficient solution was comparable with that reported in literature for A. thaliana shoots (51 mg kg⁻¹ DW) (Peer et al. 2003). Moreover, according to Helfenstein et al. (2015), there were significant differences among treatments, with leaf Zn concentration reflecting the dose in the...
nutrient solution. Co-administration of SA significantly affected leaf Zn content in plants with Zn-sufficient or Zn-excess solutions, with a slight increase in plants that had received the physiological dose and a considerable reduction in the other Zn treatments. Indeed, SA, in addition to being a hormone involved in plant defence responses, also affects nutrient element uptake (Lu et al. 2018; Mabrouk et al. 2019; Sharma et al. 2020). Some authors found that exogenous application of SA reduced Zn content in leaves (Wang et al. 2011), whereas other studies found no significant change in the mineral concentration (Shi & Zhu 2008), indicating that the precise role of SA, as well as its mechanism of action on the regulation of nutrient uptake, remain unclear. These differences in leaf Zn concentration in plants supplied with the different Zn doses, with or not SA, are related to differences in some parameters subsequently investigated.

The first parameter was pigment content, the second was lipid peroxidation. These analyses were carried out to highlight plant response to oxidative stress generally induced by nutrient imbalances, both Zn deficiency and excess. The carotenoid content was evaluated for potential role as an efficient antioxidant that protects the photosynthetic apparatus (Strzalka et al. 2003). A reduction in carotenoids with Zn deficiency or excess, and simultaneous reduction in chlorophyll, enhances the accumulation of reactive oxygen species (ROS), as reported in plants such as mandarin orange, red cabbage and tea (Subba et al. 2014).

The reduced chlorophyll content observed in Arabidopsis under both Zn-deficient and Zn-excess is similar to that reported in the literature. Indeed, Zn is involved in the development and functioning of chloroplasts (Broadley et al. 2007; Hansch & Mendel 2009; Sharma et al. 2013) and reduced chlorophyll synthesis, altered chloroplast structure and inhibition of photosynthesis are known effects of Zn deficiency (Chen et al. 2008; Fei et al. 2016; Roosta et al. 2018). Also, high Zn concentration can be phytotoxic, causing chlorosis and degradation of chloroplasts (Chaney 1993). On the other hand, Zn affects the uptake and transport of other micronutrients, such as magnesium and iron, which are directly involved in chlorophyll production (Chaney 1993). Thus, plants treated with excess Zn show iron deficiency (Haydon et al. 2012; Shannugam et al. 2012; Briat et al. 2015). Wang et al. (2016) found, in A. thaliana Col-0, that carotenoid content was not affected by Zn treatments at levels similar to those used in our experiments. Thus, the protective role of Zn as an antioxidant in photosynthesis varies with plant species and Zn dose.

Addition of SA significantly affected both chlorophyll and carotenoid concentration, increasing them only in plants given Zn excess. Similar results were reported in Tagetes erecta L., where the positive effect of SA co-supply on the level of chlorophyll and carotenoids increased proportionally with the increasing dose of the micronutrient (Choudhary et al. 2016). This positive effect could be related to the SA-mediated activation of antioxidant enzymes and biosynthesis of secondary metabolites with stress-protective roles (Kim et al. 2009; Al-Whaibi et al. 2012; Sadeghi et al. 2013).

For lipid peroxidation, as a marker of oxidative stress, we measured MDA rather than the concentration of H$_2$O$_2$ or the superoxide ion, which are transient and therefore poorly suited to assess oxidative damage after 7 days of Zn deficiency or...

**Fig. 5.** PDF 1.2 gene transcript accumulation in leaves of Arabidopsis thaliana Col-0 plants supplied with water solution of zinc sulphate at 2 µM (zinc-sufficiency), 25 µM (zinc-excess) or water solution without zinc sulphate (zinc-deficiency), and the same solutions with 0.1 mg l$^{-1}$ salicylic acid (SA), at 5 days post-inoculation with conidial suspension of Botrytis cinerea. Semi-quantification of mRNA levels loaded in each line was performed by co-amplification and normalization with an internal standard (Elongation factor-1 α; EF 1-α). Relative intensities of signals (mean ± SE) were measured using image analysis software ImageJ. Each experiment was independently repeated three times, with three plants per treatment and two leaves per plant. Data were submitted to two-factor (zinc dose × SA treatment) ANOVA. Different letters indicate significant differences ($P \leq 0.05$; Duncan multiple range test). A representative gel is shown below each histogram.
The increased content of MDA in Zn-deficient solution indicated oxidative stress. Production of ROS under Zn-deficiency is well-known, and due to iron-mediated free radical formation (Cakmak 2000). Indeed, leaf iron accumulation promotes ROS formation, especially the hydroxyl radical (OH), leading to oxidative damage to cellular components in Zn-deficient plants (Cakmak 2000; Andresen et al. 2018). Plants can reduce ROS damage by activating detoxifying enzymes, such as superoxide dismutase (SOD; EC 1.15.1.1), ascorbate peroxidase (APX; EC 1.11.1.11), catalase (CAT; EC 1.11.1.6), peroxidase (POX; EC1.11.1.7) and glutathione reductase (GR; EC 1.6.4.2) (Bowler et al. 1992; Celik & Atak 2012). Mitigation of oxidative stress is closely related to the role of SOD, a group of rapid enzymes present in most cell compartments (Alsher et al. 2002). Reduced activity of Cu/Zn-SOD, a SOD that binds both copper and Zn as cofactors, has been reported in several species under Zn deficient conditions (Sharma et al. 2004; Tewari et al. 2008, 2019; Saibi & Brini 2018), and results in increased MDA and H$_2$O$_2$ content (Sharma et al. 2004). Differences in plant antioxidant capacity and oxidative stress induction have also been reported under Zn-excess conditions (Jain et al. 2010; Feigl et al. 2015). However, in our study, 25 µM Zn did not affect the MDA content. Effectively, the Zn concentration used in our experiments, although several times higher than the physiological level, was lower than levels reported in the literature (50–800 µM) as able to mimic Zn excess (Jain et al. 2010; Feigl et al. 2015). Thus, the Zn concentration here used may be too low to determine significant accumulation of ROS, but sufficient to induce antioxidant systems that provide an effective counter to oxidative stress. However, the purpose of our work was to assess the impact of different concentrations of Zn in defence against fungal pathogens, without inducing excessive toxicity.

In plants supplied with Zn-deficient solution, addition of SA significantly reduced oxidative stress by lowering MDA to the control value, thus demonstrating the protective role of SA against ROS accumulation, as previously reported for photosynthetic pigments.

Here, the different Zn concentrations also affected responses to both necrotrophic and biotrophic fungal pathogens. The higher susceptibility of A. thaliana both under Zn deficiency and Zn excess to the necrotrophic pathogen B. cinerea, with Zn deficiency having a larger impact, could be related to the induction of oxidative stress in Zn-deficient plants, where the increase in lipid peroxidation and subsequent cellular damage benefits the necrotrophic fungus. Indeed, it has been reported...
that *B. cinerea* bypasses the oxidative burst of the host plant and exerts virulence directly proportional to the intensity of the host defence response (Tudzynski & Kokkelink 2009). Moreover, Govrin & Levine (2000) found that, during its infection process, *B. cinerea* directly causes cell death and induces a hypersensitive response (HR) in *Arabidopsis* and that *Arabidopsis* HR-deficient mutants are resistant to *B. cinerea*.

Resistance to necrotrophs, including *B. cinerea*, is usually associated with activation of the JA-resistance pathway, as highlighted by the increased transcription of JA-responsive genes, such as plant defensins (Penninckx et al. 1998; Sham et al. 2019). In the hyperaccumulator plant *Thlaspi caerulescens* J. & C. Presl and *Arabidopsis halleri* O’Kane and Al-Shehbaz, Zn deficiency and/or Zn excess strongly induce the expression of the PDF gene family (Mirouze et al. 2006; van de Mortel et al. 2006). However, in *A. thaliana* both Zn deficiency and Zn excess reduced expression of PDF genes (van de Mortel et al. 2006). Similarly, in the present work, a weak, although not significant, decrease in *PDF1.2* transcripts was observed both in Zn deficient and Zn excess plants, in comparison with control, which was supplied with a physiological Zn dose. The lack of *PDF1.2* induction could at least partially explain decreased susceptibility to the fungus observed here in plants that received an unbalanced Zn supply. Co-supply with SA greatly increased expression of *PDF1.2* transcripts both in Zn-deficient and Zn excess plants. The observed induction of *PDF1.2* after SA treatment may seem surprising, since *PDF1.2* induction is mainly linked to the JA/ET resistance pathways (Pangesti et al. 2016) that are antagonized by the SA resistance pathway. However, synergism has been reported for SA and JA/ET defence pathways (Beckers & Spoel 2006; Mur & Kenton 2006), and recent studies demonstrated that SA produced during early stages of the effect-triggered immunity (ETI) is a necessary signal not for suppression but rather for stimulation of JA signalling (Liu et al. 2016). Probably, the role of SA as a signal triggering the antioxidant systems is particularly effective where the level of oxidation is high, as in Zn-deficient plants.

With regard to *G. cichoracearum*, increased susceptibility to the fungus was observed with increasing Zn dose. The lower susceptibility of plants supplied with Zn-deficient solution could be due to the higher oxidative stress, making the cellular environment unsuitable for this fungus with a biotrophic lifestyle. Indeed, it is well known that ROS accumulation typical of the HR response is specifically associated with plant resistance to biotrophic pathogens (Balint-Kurti 2019). The higher susceptibility of plants supplied with Zn excess, might indicate that the used concentration (25 µM) did not limit the development of *G. cichoracearum*. Moreover, the more severe infection could be related to the lack of oxidative burst caused by activation of antioxidant systems in the presence of Zn concentrations higher than the physiological concentration. This response to Zn excess may have weakened the defences induced by the pathogen, such as ROS accumulation and related activation of defence genes. Indeed, it has been reported that, in comparison with the non-hyperaccumulator *A. thaliana*, in hyperaccumulator *Noccaea caerulescens* (C. Presl) a high Zn concentration resulted in reorganization of the defence pathway against *Pseudomonas syringae pv. maculicola*, with the loss of the oxidative burst and downstream responses, such as induction of SA-responsive pathogenesis-related (PR) genes (Fones et al. 2013). This supports our results, where SA addition to the nutrient solution significantly reduced infection by *G. cichoracearum* at all applied Zn doses.

**Fig. 7.** Callose deposition (%) in digitalized photos in leaves of *Arabidopsis thaliana* Col-0 plants supplied with water solution of zinc sulphate at 2 µM (zinc-sufficiency) or 25 µM (zinc-excess) or water solution without zinc sulphate (zinc-deficiency), and the same solutions with 0.1 mg l⁻¹ salicylic acid (SA), 7 days post-treatment. The experiment was independently repeated three times, with three plants per treatments, two leaves per plant and two photographs taken at random for each leaf. Data from three experiments were submitted to two-factor (zinc dose × SA treatment) ANOVA. Each column represents mean of 36 replicated photos per treatment ± SE. Different letters indicate significant differences (*P* ≤ 0.05; Duncan multiple range test). Bottom: representative digitalized areas of callose localization in leaves after aniline blue staining (blue fluorescence). Bar: 200 µm.
The critical role of SA as a hormonal signal for defense against biotrophic pathogens, such as Golovinomyces spp. (Ding & Ding 2020), was also confirmed by its effect on callose formation, which is induced in response to fungal infection (Jacobs et al. 2003). We have shown that different doses of Zn did not influence the accumulation of callose, which was instead strongly stimulated by SA supply.

CONCLUSION

This work confirms the key role of Zn in plant metabolism and defence, and demonstrates that deviation from the physiological concentration, both deficiency and excess, can significantly affect plant physiology and response to fungal infections, varying in relation to the pathogen lifestyle. Hence, Zn fertilization of crops in order to supplement this micronutrient in the human diet should be carefully evaluated because it can determine the success or failure of the crop, as well as the function of stress factors with which it might interact. From a practical point of view, the effect of mineral nutrition on plant–pathogen interactions should be investigated species-by-species and pathogen-by-pathogen so as to use the appropriate mineral nutrition as a plant protection measure.

REFERENCES

Alloway B.J. (2008) Zinc in soils and crop nutrition, 2nd edn. IZA and IFA, Brussels, Belgium and Paris, France, pp 139.
Asher R.G., Erturk N., Heath L.S. (2002) Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. Journal Experimental Botany, 53, 1331–1341.
Al-Wahabi M.H., Siddiqui M.H., Brashah M.O. (2012) Salicylic acid and calcium induced protection of wheat against salinity. Protoplasma, 249, 769–778.
Andresen E., Peiter E., Kupper H. (2018) Trace metal metabolism in plants. Journal Experimental Botany, 69, 909–954.
Arnon D.I. (1949) Copper enzymes in isolated chloroplasts: polyphenoloxidase in Beta vulgaris. Plant Physiology, 24, 1–15.
Balint-Kurti P. (2019) The plant hypersensitive response: concepts, control and consequences. Molecular Plant Pathology, 20, 1163–1178.
Beckers G.J., Spoel S.H. (2006) Fine-tuning plant defence signalling: salicylate versus jasmonate. Plant Biology, 8, 1–10.
Boller C., Montagu M.V., Inze D. (1992) Superoxide dismutases (SODs) in controlling oxidative stress in plants. Trends in Plant Science, 7, 523–529.
Cakmak I. (2000) Possible roles of zinc in protecting plant cells from damage by reactive oxygen species. New Phytologist, 146, 185–205.
Celik O., Atak C. (2012) The effect of salt stress on antioxidative enzymes and proline content of two Turkish tobacco varieties. Turkish Journal of Biology, 36, 339–356.
Chaney R.L. (1993) Zinc phytotoxicity. In: Robson A.D. (Ed), Zinc in soil and plants. Kluwer Academic, Dordrecht, the Netherlands, pp 135–150.
Choudhary A.M., Bola P.K., Moond S.K., Dhayal M. (2015) Effect of foliar application of zinc and salicylic acid on growth, flowering and chemical constituents of navel orange (Citrus sinensis). Journal of Integrative Agriculture, 15, 803–811.
Coleman J.E. (1998) Zinc enzymes. Current Opinion in Chemical Biology, 2, 222–234.
Ding P., Ding Y. (2020) Storries of salicylic acid: a plant defense hormone. Trends in Plant Science, 25, 549–565.
Duffy B.K. (2007) Zinc and plant diseases. In: Datnoff L.E., Elmer W.H., Huber D.M. (Eds), Mineral nutrition and plant disease. APS Press, St. Paul, MN, USA, pp 155–177.
Es-sbihi F.Z., Hazzoumi Z., Amrani J.K. (2020) Effect of salicylic acid foliar application on growth, glandular hairs and essential oil yield in Sabria officinalis L. grown under zinc stress. Chemical Biological Technologies, Agriculture, 7, 26.
Fei X., Fu X.Z., Wang N.Q., Xi J.L., Huang Y., Wei Z., Ling L.L., Peng L.Z. (2016) Physiological changes and expression characteristics of ZIP family genes under zinc deficiency in navel orange (Citrus unshiu). Journal of Integrative Agriculture, 15, 803–811.
Feil G., Lehotai N., Molnár Á., Ördög A., Rodriguez-Ruiz M., Palma J.M., Dröge-Winkler P., Ederli D., Kollert Z. (2015) Zinc induces distinct changes in the metabolism of reactive oxygen and nitrogen species (ROS and RNS) in the roots of two Brassica species with different sensitivity to zinc stress. Annals of Botany, 116, 613–625.
Ferrari S., Plotnikova J.M., De Lorenzo G., Ausubel F.M. (2003) Arabidopsis local resistance to Botrytis cinerea involves salicylic acid and camalexin and requires ED14 and PAD2 but not SID2, ED55 or PAD4. The Plant Journal, 35, 193–205.
Fenes H.N., Eyles C.J., Bennett M.H., Smith I.A., Preston G.M. (2013) Uncoupling of reactive oxygen species accumulation and defence signalling in the metal hyperaccumulator plant Noccaea caerulescens. New Phytologist, 199, 916–924.

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AUTHOR CONTRIBUTIONS

MQ and LE contributed to conceptualization, methodology, analysis, investigation, data curation, writing and funding acquisition. ET and RDA contributed to methodology and analysis of data related to zinc level in leaves.

CONFLICT OF INTEREST

All authors confirm that they have no conflict of interest to declare.

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Gondor O.K., Pál M., Darók É., Janda T., Szalai G. (2016) Salicylic acid and sodium salicylate alleviate cadmium toxicity to different extents in maize (Zea mays L.). PLoS One, 11, e0160157.

Govrin E.M., Levine A. (2000) The hypersensitive response facilitates plant infection by the necrotrophic pathogen Botrytis cinerea. Current Biology, 10, 751–757.

Hansch R., Mendel R.R. (2009) Physiological functions of mineral micronutrients (Cu, Zn, Mn, Fe, Ni, Mo, B). Current Opinion in Plant Biology, 12, 259–266.

Haydon M.J., Kaqachi M., Wirtz M., Hîlmer S., Hell R., Kramer U. (2012) Vascular nicotianamine has critical and distinct roles under iron deficiency and for zinc sequestration in Arabidopsis. The Plant Cell, 24, 724–737.

Heath R.L., Packer L. (1968) Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Archives of Biochemistry and Biophysics, 125, 189–198.

Helfenstein J., Pawlowski M.L., Hill C.B., Stewart J., Lagos-Kutz D., Bowen C.R., Frossard E., Hartman R., Kramer U. (2012) Vacuolar nicotianamine has homeostasis of zinc, copper, and nickel in plants. In: Marschner P. (Ed), Marschner's mineral nutrition of higher plants. Academic Press, London, UK, pp 283–298.

Jacob A.K., Lipka V., Burton R.A., Pantruga R., Strizhov N., Schule-Zeltner P., Fincher G.B. (2003) An Arabidopsis callose synthase, GLS1, is required for wound and papillary callose formation. The Plant Cell, 15, 2503–2513.

Jain R., Srivastava S., Solomon S., Shrivastava A.K., Chandra A. (2010) Impact of excess zinc on growth parameters, cell division, nutrient accumulation, photosynthetic pigments and oxidative stress of sugarcane (Saccharum spp.). Acta Physiologica Plantarum, 32, 979–986.

Jiang Z., Dong X., Zhang Z. (2016) Network-based comparative analysis of Arabidopsis immune responses to Golovinomyces orontii and Botrytis cinerea infections. Scientiﬁc Reports, 6, 19149.

Kraemer U., Clemens S. (2005) Function and homeostasis of zinc, copper, and nickel in plants. In: Tamaz M.I., Martinova E. (Eds), Topis in current genetics 14. Springer, Berlin, Germany, pp 215–271.

Lichtenthaler H.K., Wellburn A.R. (1985) Determination of total carotenoids and chlorophylls a and b of leaf in different solvents. New Phytologist, 98, 11–13.

Liu L., Sonbol M., Huo B., Gu Y., Withers J., Wu M. (2004) Identifying model crop species for the management of soil zinc deficiency. Journal of Soil Science and Plant Nutrition, 5, 1006–1017.

Mandal S., Mallick N., Mitra A. (2009) Salicylic acid-induced resistance to Fusarium oxysporum f. sp. lyocercyti in tomato. Plant Physiology and Biochemistry, 47, 642–649.

Micali C., Góllner K., Humphry M., Consonni C., Panstruga R. (2008) The powdery mildew disease of Arabidopsis; a paradigm for the interaction between plants and biotrophic fungi. The Arabidopsis Book, 6, e0115.

Mirona M., Sels J., Richard O., Czernecki P., Loubet S., Jacquier A., Francois E.L.A., Cammue B.P.A., Lebrun M., Berthoumieu M., Arques L. (2006) A putative novel role for plant defensins: a defense from the zinc hyperaccumulating plant, Arabidopsis halleri, confers zinc tolerance. The Plant Journal, 47, 329–342.

Moffa P., de Miranda J., Smita L., Tiwari R.K., Khurana D., Kumar P., Tagore S.K., Jiang Z., Dong X., Zhang Z. (2016) Network-based analysis of multiple environmental stresses identifies RAP2.4 gene associated with Arabidopsis immunity to Botrytis cinerea. The Journal of Experimental Biology, 219, 1701–1709.

Murphy A.S., Salata D.E. (2003) Identifying model crop species for the management of soil zinc deficiency. Journal of Soil Science and Plant Nutrition, 5, 1006–1017.

Nadimpalli R., Venkataraman S.P., Tewari R.K., Kumar P., Tewari R.K. (2004) Early signs of oxidative stress in wheat plants subjected to zinc deficiency. Journal of Plant Nutrition, 27, 451–463.

Qi J., Zhu Z. (2008) Effects of exogenous salicylic acid on manganese toxicity, element contents and antioxidative system in cucumber. Environmental and Experimental Botany, 63, 317–326.

Quaglia, Troni, D’Amato, Ederli (2011) Zinc in crop production and abiotic stress tolerance in plants: an overview. New Phytologist, 189, 262–266.

R., Kramer U. (2012) Vascular nicotianamine has homeostasis of zinc, copper, and nickel in plants. In: Marschner P. (Ed), Marschner's mineral nutrition of higher plants. Academic Press, London, UK, pp 283–298.

Sharma A., Patni B., Shankhdhar D., Shankhdhar S.C. (2013) Zinc as an indispensable micronutrient. Physiology and Molecular Biology of Plants, 19, 11–20.

Sharma A., Sidhu G.P.S., Araniti F., Bala A.S., Shahzad B., Tripathi D.K., Brestic M., Skalicky M., Landi M. (2020) The role of salicylic acid in plants exposed to heavy metals. Molecules, 25, 540.

Sharma P.N., Kumar P., Tewari R.K. (2004) Early signs of oxidative stress in wheat plants subjected to zinc deficiency. Journal of Plant Nutrition, 27, 451–463.

Sharma A., Patni B., Shankhdhar D., Shankhdhar S.C. (2013) Zinc as an indispensable micronutrient. Physiology and Molecular Biology of Plants, 19, 11–20.

Sharma A., Sidhu G.P.S., Araniti F., Bala A.S., Shahzad B., Tripathi D.K., Brestic M., Skalicky M., Landi M. (2020) The role of salicylic acid in plants exposed to heavy metals. Molecules, 25, 540.
zinc deficiency-induced oxidative stress in Zn-efficient maize plants. *Journal of Soil Science & Plant Nutrition*, **182**, 701–707.

Tudzynski P., Kokkelink L. (2009) *Botrytis cinerea*: molecular aspects of a necrotrophic life-style. In: Iacobellis N.S., Collmer A., Hutcheson S.W., Mansfield J.W., Morris C.E., Murillo J., Schaad N.W., Stead D.E., Surico G., Ullrich M. (Eds), *The mycota*. Springer, Berlin, Germany, pp 29–50.

USEPA (United States Environmental Protection Agency) (1996) EPA method 3052B, microwave assisted acid digestion of siliceous and organically based matrices. In: *Test methods for evaluating solid waste, physical/chemical methods*, USEPA, Washington DC, USA.

Wang C., Zhang S., Wang P., Hou J., Qian J., Ao Y., Lu J., Li L. (2011) Salicylic acid involved in the regulation of nutrient elements uptake and oxidative stress in *Vallisneria natans* (Lour.) Hara under Pb stress. *Chemosphere*, **84**, 136–142.

Wang X., Sager R., Cui W., Zhang C., Lu H., Lee J.Y. (2013) Salicylic acid regulates plasmodesmata closure during innate immune responses in *Arabidopsis*. *The Plant Cell*, **25**, 2315–2329.

Wang X., Yang X., Chen S., Li Q., Wang W., Hou C., Gao X., Wang L., Wang S. (2016) Zinc oxide nanoparticles affect biomass accumulation and photosynthesis in *Arabidopsis*. *Frontiers in Plant Science*, **6**, 1243.

Williamson B., Tudzynski B., Tudzynski P., Van Kan J.A.L. (2007) *Botrytis cinerea*: the cause of grey mold. *Molecular Plant Pathology*, **8**, 561–580.

Wong K.W., Yap C.K., Nulit R., Omar H., Aris A.Z., Cheng W.H., Latif M.T., Leow C.S. (2019) Zn in vegetables: a review and some insights. *Integrative Food Nutrition and Metabolism*, **6**, 1–7.

Yoon H.K., Muhammad H., Abdul L.K., Chae I.N., Sang M.K., Hyun H.H., In Jung L. (2009) Exogenous application of plant growth regulators increased the total flavonoid content in *Taraxacum officinale* Wigg. *African Journal of Biotechnology*, **8**, 5727–5732.

Zeng H., Xia C., Zhang C., Chen L.-Q. (2018) A simplified hydroponic culture of *Arabidopsis*. *Bio-Protocol*, **101**, e3121.