REVIEW

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Arabidopsis root growth and development under metal exposure presented in an adverse outcome pathway framework

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Abstract

Due to human activities, soils become more and more polluted with metals, which imposes risks for human health and wildlife welfare. As most of the metals end up in the food chain through accumulation in plants, we need to establish science-based environmental criteria and risk management policies. To meet these necessities, a thorough understanding is required of how these metals accumulate in and affect plants. Many studies have been conducted towards this aim, but strikingly, only a few entries can be found in ecotoxicological databases, especially on *Arabidopsis thaliana*, which serves as a model species for plant (cell) physiology and genetic studies. As experimental conditions seem to vary considerably throughout literature, extrapolation or comparison of data is rather difficult or should be approached with caution. Furthermore, metal-polluted soils often contain more than one metal, yet limited studies investigated the impact of metal mixtures on plants. This review aims to compile all data concerning root system architecture under Cu, Cd and Zn stress, in single or multi-metal exposure in *A. thaliana*, and link it to metal-induced responses at different biological levels. Global incorporation into an adverse outcome pathway framework is presented.

KEYWORDS
Arabidopsis thaliana, cadmium, copper, pollution, root development, root responses, zinc

1 | INTRODUCTION

One of the global pollution problems from the last decades is the accumulation of metals in the food chain due to anthropogenic activities such as the expansion of industrial areas, mine tailing, improper metal waste disposal and use of fertilizers and pesticides. Hence, metal concentrations rise in soils of both rural and urban environments, and these cause risks to human health, plants, animals and even to whole ecosystems (D'Amore, Al-Abed, Scheckel, & Ryan, 2005; Wuana & Okieimen, 2011). In addition, crops growing on metal-contaminated soil have reduced growth and yield, which directly impacts the economy (Grytsyuk, Arapis, Perepelyatnikova,

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Ivanova, & Vynograds'ka, 2006). As a consequence, the recognition of metals as environmental pollutants has triggered research on their propagation in the food chain and on their toxic effects. Detailed information has been gathered on the origin of metal pollutants, their distribution and speciation in the environment, the different paths of uptake by organisms and the mechanisms of toxicological impact (Asati, Pichhode, & Nikhil, 2016). For the development of effective and science-based strategies to address this growing problem, a fundamental understanding of the underlying mechanisms is essential. On one hand, it is needed for imposing adequate environmental criteria and for establishing risk management policies. On the other hand, it is crucial for the creation and optimization of possible remediation methods and technologies, and for pollution prevention initiatives (Xu, Wu, Han, & Li, 2017).

Seventeen elements, such as iron (Fe), copper (Cu) and zinc (Zn) (Gallego et al., 2012), are considered to be essential, either as micro- or macronutrients depending on their concentrations (Printz, Lutts, Hausman, & Sergeants, 2016). Despite this, they can become phytotoxic when present in (too) high concentrations and the extent of the effects does not only depend on the external and internal concentration but also on the bioavailability and internal processing, interactions with other trace metals, exposure time and frequency. Phytotoxicity affects different processes on multiple biological levels, ranging from changes in gene expression, over alterations in the cellular organization to differences in the morphologies of whole organs (Lequeux, Hermans, Lutts, & Verbruggen, 2010; Mithofer, Schulze, & Boland, 2004; Peto et al., 2013; Sofo et al., 2013; Yuan, Xu, Liu, & Lu, 2013). However, the ability of plants to cope with toxic metal concentrations and to maintain their growth is not well understood (Gomez-Sagasti, Barrutia, Ribas, Garbisu, & Becerril, 2016). Cadmium (Cd), Cu and Zn often occur in contaminated soils at elevated concentrations and they may even cause stress at relatively low concentrations (Kabata-Pendias & Mukherjee, 2007) but they do not necessarily affect plant growth at these levels. When their concentrations exceed a tolerance threshold, their effect becomes prevalent, indicating that plants can cope with metal stress to a certain extent.

Many studies have investigated the effect of single metal exposures. However, the number of studies that investigated multi-metal exposure is limited (Bochicchio et al., 2015; Gomez-Sagasti et al., 2016; Sofo et al., 2013). Nonetheless, metal-polluted sites are often polluted with multiple metals (Xu et al., 2017). Using their root system, plants take up essential trace metals, such as Zn and Cu from the soil, but they also take up nonessential metals, such as Cd (Cataldo & Wildung, 1978; Wuana & Okieimen, 2011). Risk assessments, even by modern legislations such as Regulation for Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), are usually performed on single metal exposure and thus fully ignore the mixture effects (Backhaus & Faust, 2012). Furthermore, it is well established that the combination of different chemicals can produce significant toxic effects, even though they may cause no or very limited observable effects when applied individually (Moyson, Town, Joosen, Husson, & Blust, 2019). There is thus a need to understand how metals act together in mixtures and how these should be handled

in a regulatory risk assessment context (Backhaus & Faust, 2012). Therefore, the development of efficient and science-based analytical and modelling approaches for interpreting and predicting single metal and metal-mixture toxicity in plants is required.

Multiple concepts can be used for modelling mixture toxicity: concentration addition considers that chemicals with a similar mode of action can be replaced by equivalent amounts, whereas the response addition concept is based on the assumption that the metals have a different mode of action (Versieren, Smets, De Schamphelaere, Blust, & Smolders, 2014). However, both do not consider potential interactions of the components within a mixture. For ecotoxicological assessments, metal speciation data can be used as input for toxicity predicting models, like the biotic ligand model, which includes the effect of ion competition (Qiu, Versieren, Rangel, & Smolders, 2016; Tipping & Lofts, 2015), and the Chemistry of the Uplands Model-Annual Metals, which is able to predict mixture effects in soils with various properties (Rieder et al., 2014). To even complicate things further, there is a large variation in sensitivity upon exposure to metals among plant species, as can be seen from the lowest observed effect concentrations/levels (LOEC/LOEL) deposited in the ECOTOXicology knowledgebase (https://cfpub.epa.gov/ecotox/) (Figure 1, ETX 2.1).

Interestingly, although several reports of metal-treated _Arabidopsis thaliana_ plants are present in the literature, data on this model species are mostly lacking in the ECOTOXicology database. For animal systems, the adverse outcome pathways (AOP) concept for explaining metal toxicity is generally recognized as it provides a linkage between a molecular initiating event (e.g., a molecular interaction between a toxic compound and a specific biomolecule) and an adverse outcome, via subsequent key events, through different levels of biological organization relevant to risk assessment (Ankley et al., 2010; Vinken et al., 2017). It is important that these key events make sense from a physiological and biochemical perspective. AOP studies can be used to identify knowledge gaps and they are especially important for risk assessment. This approach showed that in fish, for example, Cd exposure leads to growth reduction due to increased metabolic demands and not due to a reduction in food acquisition (Groh et al., 2015). This AOP concept is largely missing in the plant field, although AOPs offers a powerful approach to organize and assess the available knowledge on toxicity and responses. In addition, once installed, AOPs can be broadly applied to multiple metals that are known to induce similar (growth) effects. Therefore, AOPs are useful as a central framework that can be updated when novel data becomes available and they can support risk assessment based on mechanistic reasoning.

Since the root is the first plant organ to come into contact with polluted soil, this review focuses on the effects of Cu, Zn and Cd on the plant root system architecture of _A. thaliana_. On a macroscale, root system architecture describes the organization of the primary and lateral roots, and accessory roots where they are present (Lynch, 1995; Smith & De Smet, 2012). This 3D organization of roots influences the soil volume that is sampled by the roots, it is therefore a key parameter for the uptake efficiency of water and nutrients and is highly plastic towards changes in the rhizosphere (Morris et al., 2017). On a microscale, root hairs are included in this root
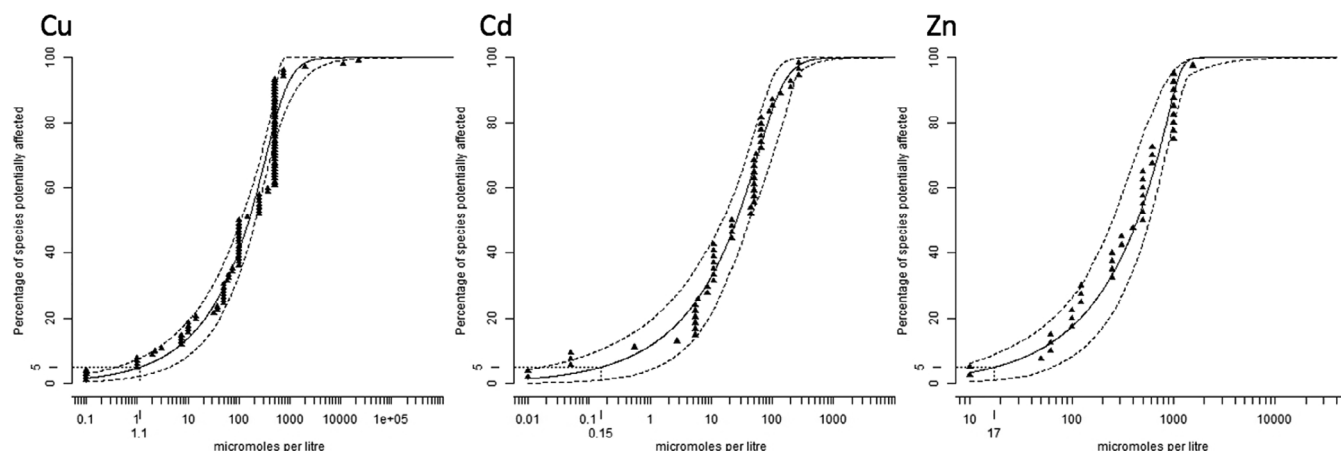
FIGURE 1 Species sensitivity distribution from the ECOTOX database for Cu, Cd and Zn for plant species on the basis of LOEC/LOEL. On the x-axis metal concentration is shown where 5% of the species is possibly affected, calculated by the program ETX version 2.1 (HC5 concentrations: Cu 1.1 μM, Cd 0.15 μM, Zn 17 μM)

system architecture, as they aid the uptake of water and nutrients by massively increasing the root's absorptive surface (Leitner et al., 2010; Vandamme et al., 2013). Being a model plant, the development of *Arabidopsis*' root system is very well described (Dolan, Janmaat, & Willemsen, 1993; Verbelen, De Cnodder, Le, Vissenberg, & Baluska, 2006). The root meristem of *A. thaliana* reaches its final size 5 days after germination and the size of the meristem is determined by the balance of two processes, that is, cell division and cell differentiation, which are both under tight hormonal regulation by auxin and cytokinin (Beemster & Baskin, 2000). In recent years, some studies were undertaken to assess the effect of single metal exposure and combinations of two metals on plant development, by studying general endpoints such as growth, biomass production, photosynthesis efficiency, and so on. However, the exact underlying mechanisms behind the observed responses to excess metals remain largely unexplored. This makes a comparison of the effects of different metals, supplied as singles or mixtures, rather difficult. Therefore, this review presents data on the effects of exposure to Cu, Zn, Cd in a single- and multi-pollution context at the organizational level of root system architecture and links this to possible underlying modes of action. An outline of an AOP framework for metal effects in plant roots is presented and this provides the possibility to add new findings to this existing framework.

2 | DIFFERENT EXPERIMENTAL SET-UPS COMPLICATE EXTRAPOLATION OF RESULTS

Depending on soil conditions, roots optimize their ability to provide water and nutrients for the plant or to limit the uptake of undesirable substances by mechanisms spanning all systemic levels. At the morphological level, roots can alter many of their characteristics, such as the length and diameter of primary roots, the lateral root density, lateral root length and growth angle, the presence and length of root

hairs, generally described as root system architecture (Araya et al., 2014; Morris et al., 2017).

The uptake, accumulation and toxicity of trace elements in the root highly depend on the root's environment, soil composition and structure, pH and cation exchange capacity, water content and organic matter and other chemical and physical characteristics (Peralta-Videa, Lopez, Narayan, Saupe, & Gardea-Torresdey, 2009). In addition, the toxicity of metals to plants can vary significantly and correlates with metal concentration and speciation. The form in which metals are present in the soil highly determines the metal uptake and plant toxicity (Chiang, Huang, & Chang, 2007; McLaughlin, Smolders, & Merckx, 1998). However, it seems that this is currently insufficiently considered. Therefore, this issue should be taken into account when comparing experimental data to have a comparable exposure scale.

Some general patterns of metal toxicity, such as inhibition of primary root growth and alterations in lateral root density are recognized, regardless of the exposure conditions, and can become apparent at different metal concentrations or after different exposure times (Figure 2). Depending on the speciation of a given metal or group of metals in the soil, the toxicokinetics and dynamics may vary significantly (Qiu et al., 2016). In an attempt to investigate the effect of excess metals on roots of the model plant *A. thaliana*, researchers have implemented different approaches. For the analysis of root system architecture, seedlings are commonly grown on vertically positioned metal-containing half or full-strength Murashige and Skoog media (½ MS or MS, respectively), or in hydroponics for a certain period of time before the roots are used for measurements (Bochicchio et al., 2015; Lequeux et al., 2010; Pasternak, Rudas, Potters, & Jansen, 2005; Song et al., 2017; Wang, Wang, Zhao, Yang, & Song, 2015; Wojcik, Pawlikowska-Pawlega, & Tukiendorf, 2009; Wojcik & Tukiendorf, 2003; Yuan et al., 2013). Soil, being the growth medium in natural conditions, is used to provide more environmentally relevant data but is also inconvenient to visualize soil-grown roots at high resolution.
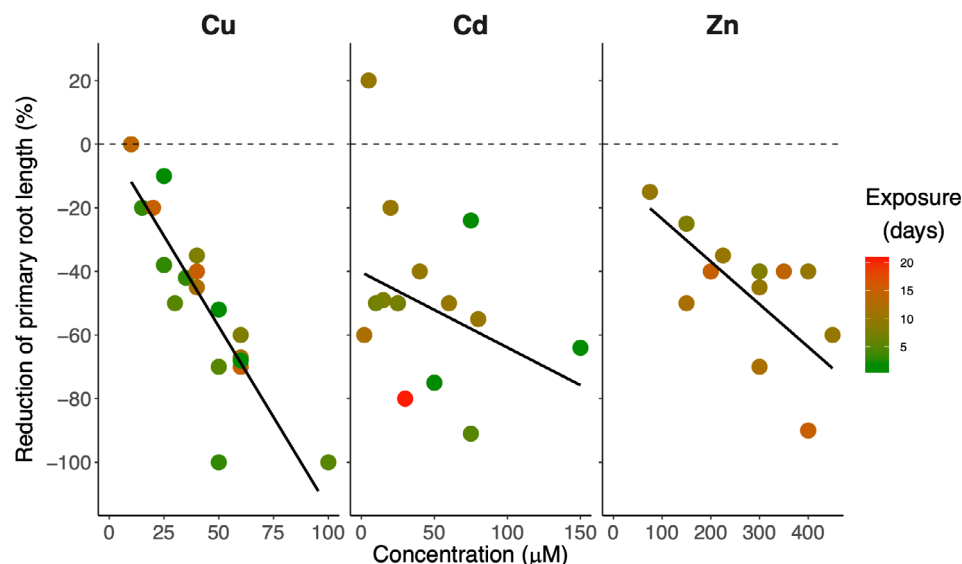
FIGURE 2 Effect of a single exposure of different trace metals on ½ MS-media on the growth of primary roots in *A. thaliana*. Data were collected from the literature (Supplementary Table S1) and combined. Note that *x*-axes are different for the individual metals. Not included in the figure is the difference of plants that were sown immediately on metal-contaminated plates or that were transferred. Cd, cadmium; Cu, copper; Zn, zinc [Colour figure can be viewed at wileyonlinelibrary.com]

This summary clearly shows that even setting up conditions for metal toxicity studies is not straightforward in terms of metal exposure (i.e., concentration, speciation and duration) and environmental growth conditions. It is therefore important to deal with the exposure to varying concentrations of trace metals using commonly used reference points, such as the NOEL/LOEL in mixtures or EC50 (or more specifically biomass, ECb50). To get a better view of the modes of action, standard sampling should be included to follow up initial effects of the metals and to study how the accumulation affects plant growth. In the following sections, the effects of metals are reviewed. To keep the text concise, each effect is compared to the control treatment for each corresponding experiment and abbreviations, such as Cu20, Cd20, Zn200 and so on, are used to express treatments with particular concentrations (20, 20 and 200 µM) of a particular metal (Cu, Cd and Zn), respectively. Regardless of the chemical form of the metal, which was administered in the exposure media ($CuSO_4$, $CdCl_2$, $ZnSO_4$, etc.), only the metal element (Cu, Cd and Zn in this case) is mentioned.

3 | INITIAL MOLECULAR EVENTS

The initial effects of trace metals on a molecular event are poorly understood. Due to the positive charge of Cu, Zn and Cd, as well as the presence of other contaminants, they are soil-bound cations or complexed in organic molecules, and they are absorbed by the roots and transported across the plant (Sofo et al., 2013). Trace metals, including Cu, Cd and Zn, can be transported across plant membranes by numerous metal transporters, such as members of the ATPase transporter family, ZRT/IRT-like protein family and the copper transporter family (Kramer, Talke, & Hanikenne, 2007). It is even known that trace metals can be cotransported through the plasma membrane (Kramer et al., 2007) but less is known on the recognition and "action" of the individual trace metals in the cell. Many abiotic stressors, including metals, alter the production of reactive oxygen species

(ROS) and induce oxidative stress (Pasternak et al., 2005; Yadav, 2010). Trace metal stresses can shift the cellular balance of free radical homeostasis into the accumulation of hydrogen peroxide (H_2O_2). Oxidation in the Fenton reaction and Haber–Weiss reaction will convert H_2O_2 to the highly reactive hydroxyl radical ($^\bullet OH$) in a metal-catalysed way (Kehrer, 2000; Mithofer et al., 2004). Together with superoxide ($O_2^{\bullet-}$) these molecules constitute the so-called ROS and are important regulators of processes as root growth and cell differentiation as well as of stress responses (Dunand, Crevecoeur, & Penel, 2007). Based on their physicochemical properties, metals will induce an oxidative challenge directly or indirectly (Cuypers, Vangronsveld, & Clijsters, 1999), and therefore, this should be integrated into an AOP framework.

Trace metal toxicity may also result from metals binding to sulphydryl and other groups leading to activity inhibition or structural distortions of proteins. Essential Cu, Ni and Zn and nonessential Cd compete for the same transmembrane carrier for their transport and can affect uptake and transport of mineral nutrients in plants in a complex way (Crowley, Wang, Reid, & Szaniszlo, 1991). In particular, the uptake of Cd ions seems to be in competition for the same transmembrane carrier with nutrients, such as K, Ca, Mg, Fe, Mn, Cu, Zn and Ni (Das, Samantaray, & Rout, 1997; Rivetta, Negrini, & Cocucci, 1997). When taken up, Cd behaves similar to Zn as they are both bivalents with a high affinity for sulphydryl groups (Sofo et al., 2013). Zinc is known to partition with Mg and to decrease enzyme activity (Ebbs & Kochian, 1997). Copper exposure revealed higher electrolyte leakage and lipid peroxidation compared to Cd (Skorzynska-Polit, Drazkiewicz, & Krupa, 2010).

4 | INITIAL PLANT ROOT RESPONSES TO CU, CD AND ZN EXPOSURE

Upon exposure to metal stress, plants activate specific detoxification mechanisms related to metal chelation and sequestration to cope with

metal phytotoxicity that are important to consider in an AOP framework (Figure 3) (reviewed in Clemens, 2001). When exposed to excess trace metals, responses differ depending on the concentration and time of exposure. Some defence mechanisms may already be present in the cell, whereas others will need some time to become activated. Glutathione (GSH) plays an important role in the defence against metal stress as it serves as a precursor of the metal-chelating phytochelatins (PCs). The enzyme phytochelatin synthase (PCS) is activated upon exposure to a number of metals, with Cd being a strong inducer, while Zn and Cu activate PCS to a lesser extent (Cobbett, 2000). It uses reduced GSH to synthesize different PC molecules. GSH and PC bind metal ions resulting in complexes that can be transported to the vacuole via tonoplast localized cation/proton exchanger or ATP-Binding Cassette transporters, thus sequestering the toxic metal ions and preventing their deleterious effects on cells (Cobbett, 2000). Weber, Trampczynska, and Clemens (2006) showed that Cd exposure induced a rapid (after 2 hr) response in glutathione S-transferase transcription in *Arabidopsis halleri*. In *A. thaliana*, transcripts of antioxidant enzymes, such as APX, CAT, CSD and GSH, were induced after 24 hr of Cu or Cd exposure (Smeets et al., 2013). Furthermore, GSH-related genes are still induced after 7 days of Cd exposure, as well as lignin and sulphate metabolism-related genes (De Mortel et al., 2008). This suggests that GSH biosynthesis is switched on and is not reduced back to control levels as long as there is trace metal contamination. Furthermore, PCs are believed to have a role in root-to-shoot metal translocation and their sequestration varies between organs within plants (Marentes & Rauser, 2007). Metallothioneins (MT) are rich in metal-binding motifs and known to be the main Cu-detoxifying molecules and they are also important in

the defence against Cd and Zn excess (Guo, Meetam, & Goldsbrough, 2008). Other amino acid chelators, including proline, glutathione, polyamines and so on, appear to play roles in metal binding, metal hyperaccumulation and metal stress defence, as well as signalling and antioxidation (Sharma & Dietz, 2006). Plants have thus developed different defence mechanisms against various trace metals. For Cd ions these are binding Cd by phytochelatins, transporting and sequestering Cd in the vacuole and for Cu and Zn this is achieved by chelating by MTs (Ernst, Krauss, Verkleij, & Wesenberg, 2008; Yadav, 2010).

When the cellular redox status is disturbed, an oxidative challenge occurs leading to signalling and/or damage (Cuypers et al., 2016). Excess Cu induces responses in roots similar to those caused by other stressors that interfere with oxidative homeostasis. Upon Cu exposure, oxidative damage, that is, lipid peroxidation is often observed. The effect of excess Cu on root growth is at least partially an effect of the disturbance in redox homeostasis, as suggested by the finding that mutants deficient in ascorbic acid, *vtc2-1* and *vtc2-3*, are more sensitive than wild type plants, and mutants with reduced ROS content showed increased cell viability upon low Cu excess (Peto et al., 2013). Cadmium and Zn, unlike Cu, have no redox activity. Nevertheless, they are known to cause oxidative stress by inducing ROS producing enzymes and by interfering with enzymes and metabolites of the antioxidative defence systems (Cho & Seo, 2005; Cuypers, Vangronsveld, & Clijsters, 2001; Gielen, Remans, Vangronsveld, & Cuypers, 2016; Opdenakker, Remans, Keunen, Vangronsveld, & Cuypers, 2012; Remans et al., 2010, 2012; Smeets et al., 2009, 2013). In addition, in this case, a metal-induced oxidative challenge leading to damage or signalling is established. It is clear that ROS are considered essential components of signal transduction
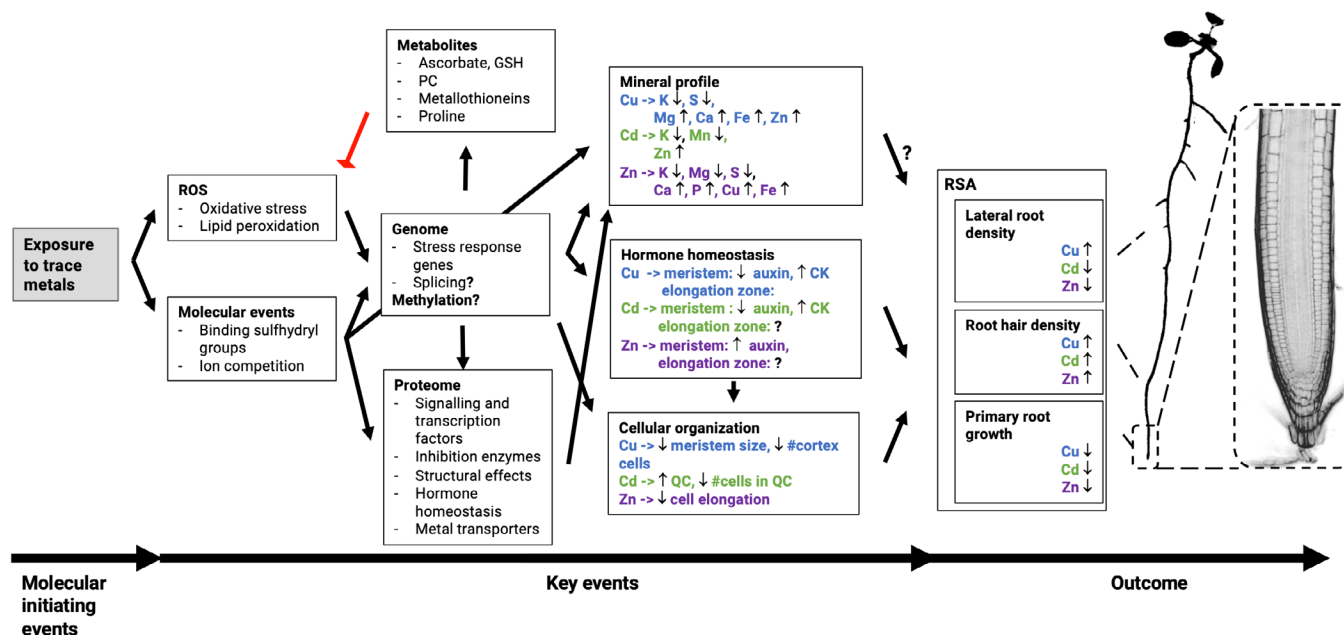

FIGURE 3 An AOP framework integrating developmental root responses as an endpoint upon exposure to Cu, Cd and Zn. Exposure to trace metals starts with initial molecular events. These then initiate responses and changes at different biological levels in the cells and organs, identified as different but linked key events. In the end, the result of these key events is the outcome, which in the case of the root is defined as the effects on the root system architecture. Cd, cadmium; Cu, copper; CK, cytokinin; GSH, glutathione; PC, phytochelatin; ROS, reactive oxygen species; RSA, root system architecture; Zn, zinc [Colour figure can be viewed at wileyonlinelibrary.com]

used by plants to respond to developmental and environmental cues (Gielen et al., 2016).

Trace metals enter plant roots by freely diffusing through the cell wall (Keyster et al., 2020). An important accumulator of heavy metals is the cell wall (Iwasaki, Sakurai, & Takahashi, 1990; reviewed by Krzeslowska, 2011), which is mainly composed of pectins and (hemi) cellulose (Gutsch et al., 2019) and which has the ability to bind heavy-metal ions in negatively charged sites, resulting in the alteration of cell wall composition (Fan et al., 2011). The induction of ROS in the apoplast causes oxidative cross-linking of cell wall components (De Cnodder, Vissenberg, Van Der Straeten, & Verbelen, 2005; Wojtaszek, 1997), which has been suggested as a possible mechanism to restrict cell growth (Knox, 1995). In response to the stress arising from trace metals, plants will increase their capacity for trace metal accumulation by elevating the amount of polysaccharides, especially pectins, which can bind trace metal ions (reviewed by Krzeslowska, 2011). When plants grow in highly contaminated soil, Ca is replaced by other metal cations, such as Cu, Cd and Zn, which indicates that they have a higher affinity for these sugar compounds (Dronnet, Renard, Axelos, & Thibault, 1996). In addition, plants synthesize callose and lipid compounds that decrease cell wall permeability and that therefore limit the amount of trace metals that can enter the protoplast. Thickening of the cell wall in leaves is possibly regulated by ethylene production (Ge et al., 2009). However, how this mechanism is regulated in roots remains poorly understood.

To acclimate to metal stress, gene expression needs to be regulated to establish a new level of cellular homeostasis to cope with metal phytotoxicity. Without any doubt, this requires a complex regulation that occurs at different levels, such as the epigenetic, transcriptomic, post-transcriptomic and post-translational levels.

5 | EFFECTS OF CU, CD AND ZN ON METAL ACCUMULATION AND MINERAL PROFILE

To correlate growth responses to metal uptake and its effect on the mineral profile, hydroponical experimental setups are used as they facilitate elemental measurements in roots. Roots accumulate a significant amount of the Cu and Cd already after 1 day proportional to its concentration in the medium (Jozefczak et al., 2014; Lequeux et al., 2010; Połeć-Pawlak, Ruzik, Abramski, Ciurzyńska, & Gawrońska, 2005). Within the whole Cu exposure period, dynamic alterations were observed for the mineral profiles of calcium (Ca), magnesium (Mg), P, K, Fe, Zn, sulphur (S) and manganese (Mn) as well (Lequeux et al., 2010). In Cu10-treated plants, after 24 hr the content of K was found to be significantly decreased (Smeets et al., 2009). In Cd-treated roots, Zn, Mn, K and Fe contents decreased (Leskova, Giehl, Hartmann, Fargasova, & von Wiren, 2017; Smeets et al., 2009). The dynamics of Cd accumulation were thoroughly investigated in a study utilizing energy-dispersive X-ray microanalysis (Van Belleghem et al., 2007). In the cortex Cd seems to be associated with P in the apoplast and with S in the cytoplasm of cells, the latter suggesting

phytochelatin (Weber et al., 2006) involvement, which is a known mechanism of metal detoxification in plants. In roots, like in shoots, excess Zn can be sequestered in the vacuoles by the action of membrane transporter proteins like metal tolerance protein 1 (MTP1) (Desbrosses-Fonrouge et al., 2005). Furthermore, with the increase of Zn content in Zn-treated roots, lower Mn and Fe levels were reported (Leskova et al., 2017). A short-term exposure (24 hr) led to decreased Mg, K and S contents in roots and an increase in the levels of Ca, P, Cu and Fe (Opdenakker et al., 2012). It is important to mention here that the toxic effects of Zn in ½ MS media were found to be strongly associated with the decreased Fe content in shoots that it causes (Shanmugam et al., 2011).

When provided as a mixture, Cu, Cd and Zn were still efficiently accumulated by roots and shoots (Sofo et al., 2013). However, the uptake of Cd in these plants was significantly lower than in plants exposed to Cd alone. The same was evident for Cu, albeit to a lesser extent, whereas Zn accumulation was not affected by the presence of the other two metals in the media. The root-to-shoot translocation efficiency for each metal was not different in mixture treatments compared to single-metal treatments (Sofo et al., 2013). In this study, however, the effects of the metals provided in doubles were not investigated, which makes it difficult to infer possible influences on the effects of one metal by another. To investigate this mutual effect, Cu and Cd were provided as single-metal treatments and mixtures in another study (Smeets et al., 2009). Besides the lesser accumulation of both metals in the mixture-treated roots as compared to the single exposures, decreases were observed for K, Mn and Zn concentrations, whereas from the single exposures only Cu caused a decrease in K. The decreased K content, that was stronger in the mixture-treated roots, is indicative of membrane leakage and might be due to lipid peroxidation that was strongly increased in the mixture treatment (Smeets et al., 2009).

6 | EFFECT OF EXCESS CU, CD AND ZN ON THE CELLULAR ORGANIZATION OF ROOTS AND HORMONAL HOMEOSTASIS

To get a clear view of the underlying mechanisms affecting root system architecture upon plant metal exposure, it is essential to investigate root development alongside other responses. Root system architecture development is largely coordinated by the action of several plant hormones (Smith & De Smet, 2012). Therefore, the altered root system architecture under metal exposure can be viewed as a response to the metal-enriched environment possibly through changes in hormone levels. To accumulate in the roots, auxin is first synthesized in shoots/leaves and then transported mainly by proteins from the PIN-FORMED (PIN) family. Cytokinins are other well-studied hormones with a recognized role in root growth regulation and plasticity. The root cap is the primary site for their synthesis and in roots cytokinins have a negative effect on growth (Aloni, Aloni, Langhans, & Ullrich, 2006). Many studies have shown that the development and maintenance of meristems are mainly controlled by crosstalk between

auxin and cytokinin, the output of which spatiotemporally dictates cell cycle activity and cell differentiation (Aloni et al., 2006; Overvoorde, Fukaki, & Beeckman, 2010; Su, Liu, & Zhang, 2011; Vieten et al., 2005). During primary root growth, the effect of environmental stimuli is imprinted on the cells located at the meristem in a way that eventually influences the development of the lateral roots and the modelling of root system architecture in general (Ingram & Malamy, 2010). For this reason, close investigation of the cellular organization at the root meristem of plants experiencing metal toxicity yields valuable insights about the effects underlying the morphological alterations, which can be linked to subcellular endpoints in an AOP framework (Table 1; Figure 3).

On solid ½ MS media, exposure to Cu20 was shown to cause no changes to the meristem size assessed as the distance from the quiescent centre to the end of the transition zone (Song et al., 2017). The

TABLE 1 A framework integrating developmental root responses as an endpoint connected to an AOP upon exposure to Cu, Cd and Zn

AOP level	Endpoints
Root system architecture and biomass	Primary root length
	Lateral root density
	Length of lateral roots
	Premature lateral root emergence
	Root hair formation
	Fresh and dry weight
ROS	Membrane and protein damage
	Altered signalling
Metabolites	Ascorbate
	Glutathione
	Phytochelatin
	Metallothionein
Cellular organization	Meristem length
	Number of cells in meristem
	Cell cycle activity
	Cell viability
	Number of cells at the quiescent centre
	Cell size
	Vacuole alterations
	Cell elongation
Hormonal homeostasis	Auxin
	Cytokinin
	Ethylene
Proteome	Signalling cascade and transcription factors
	Oxidative stress-related enzymes
	Metal transporters
Genome	For example, stress response genes, metal-ion transporters
Methylome	Methylation
Mineral profile	Mineral concentrations and homeostasis

number of cortical cells along a cell file spanning this zone was not altered either. Treatment with Cu50 for 24 hr, however, significantly reduced both meristem length and cortex cell number, suggesting changes in the cell division process (Lequeux et al., 2010). Using specific marker lines, it was shown that this effect was not due to changes in stem cell niche activity (QC25::GUS, QC46::GUS, SRC::GFP), but rather because of alterations in meristematic cell division potential (CYCB1;1::GUS) (Song et al., 2017; Yuan et al., 2013). Concentrations affecting the meristem size and cell number also negatively affected cell viability at the primary root and lateral root meristems (Lequeux et al., 2010; Peto et al., 2013). Even short-term exposure (9 hr) to higher concentrations of Cu (40 to 60 μM) induced a significant reduction of meristematic zone sizes and even of the adjacent elongation zone (Yuan et al., 2013), revealing a strong and rapid effect of excess Cu. Treating plants with excess Cu results in abnormalities in auxin distribution and abundance. For example, Cu40 and Cu60 decreased the auxin levels at columella cells but led to an increase in the root meristem and elongation zones of treated roots on ½ MS media (Yuan et al., 2013). The accumulation of auxin above the root apex was suggested to account for the enhanced formation of lateral roots and root hairs following Cu treatments, especially since auxin plays an important regulatory role in both processes (Pasternak et al., 2005; Peto et al., 2011). Another study, using hydroponics and the same marker line treated with Cu50 and Cu75 for 24 hr, showed strongly decreased auxin content in meristems, but increased auxin in the elongation zone (Lequeux et al., 2010). The genes responsible for the synthesis of the most abundant auxin in plants, IAA, were significantly downregulated in roots under Cu treatment. Changes in expression patterns of genes involved in the polar auxin transport (PIN1, AUX1 and PIN2) in primary root meristems of Cu-treated plants identified PIN1 as the only gene involved in the auxin-mediated Cu-induced growth reduction (Yuan et al., 2013). In addition, exposure to excess Cu was shown to cause a slight increase in cytokinin levels in the root cap and in the meristem at Cu50 and a significant decrease at Cu60 and Cu75 (Lequeux et al., 2010; Song et al., 2017). In contrast to these findings, Yuan et al. (2013) reported an increase of auxin levels in the meristematic zones of roots treated with up to 60 μM concentrations of Cu. These observations are particularly interesting as they can be associated with the aforementioned decrease of dividing cells in the meristem of Cu-treated plants since the production of cytokinins is known to be increased in the initial stages of mitosis. Furthermore, it was shown that cytokinin can induce the synthesis of IAA through a tryptophan aminotransferase (CKRC1) in the roots treated with excess Cu and this induction is required for proper auxin-mediated response to this metal (Song et al., 2017). Ethylene, known to stimulate root hair formation (Vissenberg, Claeijs, Balcerowicz, & Schoenaers, 2020), acts on root growth through effects on auxin biosynthesis and distribution (Markakis et al., 2012; Ruzicka et al., 2007). It was reported that Cu stress results in a slight but not significant rise in ethylene production in Cu-treated roots (Lequeux et al., 2010) and that lack of perception for this hormone in the ein2-1 mutant do not lead to altered sensitivity to Cu toxicity (Yuan et al., 2013), suggesting that ethylene is not a prime target for Cu toxicity.

Like Cu, Cd treatment did not alter stem cell niche activity. In contrast to Cu, no effect on meristematic cell division potential was observed under Cd exposure (Yuan & Huang, 2016). However, in the first 12 hr after exposure Cd managed to inhibit root meristem size due to a decrease in auxin levels. Another observation was that in the roots of plants exposed to Cd50, an enlargement of the quiescent centre was obvious. Interestingly, the enlarged quiescent centre contained fewer but larger cells. In addition, it induces an ectopic expression of the SCARECROW (SCR) gene in the stele at the level of the transition zone (Bruno et al., 2017). Treatment with Cd60 caused ectopic growth of root hairs in the root elongation zone. Concomitant to that, an enlargement of the cortical cells and an increase in the division of cells at randomly located sites in the pericycle were observed. Treatment with Cd90 led to full disruption of the meristematic cells (Brunetti et al., 2011). Plants grown on ½ MS and then transferred to hydroponics for Cd exposure showed more complex shaped and larger vacuoles in root cortex cells (Sharma et al., 2017). The effect of Cd on auxin homeostasis appears to be very similar to the effect of Cu. This suggests that both metals might have a similar mode of action in terms of interaction with components associated with auxin homeostasis. Alternatively, both of them may directly or indirectly induce the same auxin-relayed response to disturbance. Most studies show that auxin levels in the meristem are reduced under Cd treatment (Yuan et al., 2013). The same observation was made on ½ MS after Cd10 and Cd20 treatments, which abolished expression of the influx carrier *AUX1* (Sofo et al., 2017). Disturbed auxin signalling in *tir1* seedlings, lacking a functional TIR1 auxin receptor, led to decreased sensitivity to Cd. Furthermore, IAA17, which is a transcriptional repressor of auxin-responsive genes, was found to be stabilized upon Cd treatment. To further explore the underlying cause of this auxin level decline at the primary root meristem, *pin1*, *pin3* and *pin7* mutants were exposed to Cd100. The single mutants were affected by the treatment to a similar extent as the control, whereas the triple mutant *pin1pin3pin7* was less sensitive to it. This suggests that these genes have redundant roles in the Cd-induced polar auxin transport disruption (Yuan & Huang, 2016). In another study, the abundance of PIN1 was significantly reduced, no change was evident for PIN2, and PIN3 distribution was highly altered and nearly fully diminished. The protein PIN7, usually being detected in columella and in provascular cells, was weakly spread in the stele and almost absent in the columella of Cd-treated roots. In addition, also the genes encoding these proteins were found to be down-regulated (Bruno et al., 2017). All data show that Cd largely works through effects on auxin. A single report on the Cd effect on cytokinin homeostasis in roots showed lower cytokinin levels in the root cap, but significantly higher levels in both root apical meristem and above all in the transition zone (Bruno et al., 2017).

Research using iTRAQ-based proteomic analysis showed that roots treated with excess Zn had decreased amounts of V-ATPase subunits (Fukao et al., 2011). In addition, the roots of *det3-1* plants (bearing a mutation in V-ATPase subunit C) displayed a similar phenotype as wild-type Zn-treated plants. The root growth inhibition caused by excess Zn was shown to be due to disturbed cell elongation, rather than alterations in proliferation, and V-ATPase activity was suggested to play a central role in this effect (Fukao et al., 2011). Seedlings grown on ½ MS media supplemented with Zn350 had roots with lower auxin levels at the meristems of primary and lateral roots (Wang et al., 2015). When grown in ½ MS media with increasing concentration of Zn (from surface to bottom) auxin levels increased in the meristem at concentrations of 100 μM with no alteration in distribution and decreased with 200 μM concentrations (with decreased expression of *AUX1*), again with no change in distribution (Sofo et al., 2017).

When provided in the media simultaneously, Cu, Cd and Zn resulted in a more than five-fold increase in IAA levels in roots. Interestingly, Cu and Cd caused a two-fold induction each when provided alone and Zn did not have any effect (Sofo et al., 2013). Increased cytokinin levels, more specifically transzeatin and dihydro-zeatin riboside, were also observed in all treatments, except for dihydro-zeatin riboside in single Cu-treated roots. The authors suggested that this increase was not sufficient to induce root growth (as would be expected) due to the parallel elevation of auxin levels, which retarded it. Compared to the control, the ratio IAA/cytokinin was significantly altered in favour of IAA (Sofo et al., 2013).

7 | EFFECTS OF CU, CD AND ZN ON THE SEEDLING GROWTH AND ROOT SYSTEM ARCHITECTURE OF *A. THALIANA*

Models aiming to predict the toxicity of a single metal or mixture of metals usually relate the concentration of the toxicant(s) in the media to effects on endpoints at the physiological, organismal or population level (e.g., survival, growth and reproduction). For the development of environmental-regulation-integrative AOPs, the selection of the endpoints that are relevant for risk assessment is crucial. Such endpoints need to be causally related to preceding events and they need to represent outcomes that are of organismal and ecological concern (Ankley et al., 2010). Current knowledge on metal stress responses is integrated into an AOP framework to explain root growth and developmental responses under exposure to Cu, Cd and Zn (Figure 3). In addition, the reduction of biomass is an endpoint that represents a level of disturbance of the overall growth process. In general, biomass decreases with exposure to increasing metal concentrations above certain exposure levels. The biomass of roots, challenged with Cu, Cd or Zn, was shown to increase in low concentrations and decrease more severely compared to shoots with rising concentration and longer exposure (Gielen et al., 2016; Lequeux et al., 2010; Opdenakker et al., 2012; Sofo et al., 2017; Wang et al., 2015; Wojcik & Tukiendorf, 2003). Furthermore, the effect of root growth reduction only becomes noticeable after a couple of days and in a concentration-dependent manner, showing that the accumulation of stress eventually reduced growth (Bruno et al., 2017). While root fresh weight is decreased after 72 hr exposure to low doses of Cu or Cd, the fresh weight of the leaves is not (Gielen et al., 2016). This, however, does not reflect the state of the stress levels, as these are rapidly induced by Cu after 2 hr, and Cd after 24 hr even though Cd

concentrations were higher (Gielen et al., 2016). Interestingly, the deficiency of particular nutrients has been linked to alterations of root system architecture (Kellermeier et al., 2014). Thus, for setting up an AOP framework, this parameter could provide qualitatively integrative data. Few studies have been conducted so far to associate the involvement of mineral profile alterations during metal stress with the ultimate effect on root system architecture.

7.1 | Copper

On ½ MS media, low concentrations of Cu (2–5 μM) stimulate primary root growth (Bochicchio et al., 2015). At Cu10 the length of the primary root is not affected, but lateral root density increases up to 35% (Lequeux et al., 2010; Song et al., 2017). Cu also causes a dose-dependent decrease in primary root lengths at concentrations higher than 20 μM (Table S1) (Peto et al., 2011; Silva-Guzman, Addo-Quaye, & Dilkes, 2016; Yuan et al., 2013) and increases the lateral root density and premature emergence (Lequeux et al., 2010; Song et al., 2017). Root hair density increases and root hairs appear closer to the root tip (Pasternak et al., 2005; Wang et al., 2015). This redistribution of growth is thought to be a mechanism to reorganize the structure of the root and serves as a nice illustration of root growth plasticity (Pasternak et al., 2005). The precise molecular mechanisms behind this response are currently unknown.

In experiments using hydroponic methods of cultivation primary root growth-inhibiting, Cu concentrations are 10 times lower than in experiments using solid ½ MS media. For example, Cu5 significantly inhibits root growth in this setup (Lequeux et al., 2010). Even a concentration as low as 0.2 μM has been reported to severely reduce root elongation after 5 days of exposure (Toda, Koyama, & Hara, 1999). For plants initially grown on autoclaved garden soil for 7 weeks and then transferred to the test solution with the toxicant being added after 2 days, the lowest concentration causing complete growth inhibition was 150 μM. In the same experiment, exposures to concentrations from 5 to 100 μM Cu already caused dose-dependent growth retardation after 2 days. After 4 days of Cu10 treatment, primary root length was reduced by 50% (Wojcik & Tukiendorf, 2003). In another experiment, treating 2-week-old plants with Cu5 in sand media by continuously adding metal-containing Hoagland solution for 12 days, led to 42% inhibition of root growth (Sofo et al., 2013). Exposure to higher Cu concentrations (50 μM) caused lateral root density reduction, darkening and thickening together with the unnatural curving of the roots, besides a reduction in fresh weight (Drazkiewicz, Skorzynska-Polit, & Krupa, 2010).

7.2 | Cadmium

For treatments with Cd on ½ MS solid media, lethal concentrations were shown to be 300–500 μM, at which 46% and 67% of plants, respectively, did not survive after 21 days (Cho & Seo, 2005). At lower concentrations (5–80 μM) primary root growth inhibition was similar

in magnitude to the one under Cu toxicity, also including the hormetic effect at the lower concentrations (Table S1) (Leskova et al., 2017). In contrast to Cu, Cd was reported to reduce the lateral root density (Leskova et al., 2017; Sofo et al., 2017).

Experiments using hydroponics showed no effect on root length upon short exposures to Cd concentrations up to 10 μM (Cuypers et al., 2011; Smeets et al., 2013). However, 21 days of exposure to Cd5 and Cd50 caused severe primary root length reduction (Van Belleghem et al., 2007). Yet, with this set-up, Cu appears to be more toxic for primary root growth than Cd, especially at the lower range of concentrations suggesting different modes of action. In an experiment utilizing sterilized sand with Hoagland solution, the addition of Cd to the media of 2-week-old plants caused near 50% primary root length reduction and an increase in root branching, root hair density and root mean diameter. Compared to Cu5 under the same conditions, Cd10-induced higher root hair density and similar primary root length inhibition, root branching and increased root diameter (Sofo et al., 2013).

7.3 | Zinc

The primary root length-inhibiting concentrations of Zn for *A. thaliana* plants grown on ½ MS media are higher than those for Cu and Cd. On ½ MS media a dose-dependent reduction (15–60%) of primary root length was reported for plants treated with concentrations ranging from 75 to 450 μM (Leskova et al., 2017), which is consistent with other studies (Table S1) (Kim et al., 2009; Shanmugam et al., 2011; Silva-Guzman et al., 2016; Wang et al., 2015). Low Zn concentrations also induced an increase in lateral root elongation, but higher concentrations decreased this. In addition, abnormally branched root hairs were formed on excess Zn-treated roots (Fukao et al., 2011). In sterilized sand with Hoagland solution, Zn150 caused a near two-fold inhibition of primary root length of 2-week-old plants. This similar effect was caused by Cu5 and Cd10 under the same conditions (see above). Root hair density, branching and diameter were increased to a similar extent as Cu5 and Cd10 (Sofo et al., 2013). Nevertheless, treatments with up to 500 μM concentrations of Zn for 24 hr in a hydroponic set-up did not lead to any observable effects on the primary root length of *A. thaliana* (Remans et al., 2012).

7.4 | Mixtures

When added in mixtures, the effect of Cu, Cd and Zn is more severe than the effect caused by each metal alone (Yuan et al., 2013). However, the cumulative effect does not appear to be additive. In other words, primary root length reduction for multimetal-exposed plants is less than the sum of primary root length reductions for each single metal exposure. This probably is, at least partially, due to uptake competition of metals in the mixture treatment. Metal mixtures induced increased root hair and lateral root formation, but not more severely than upon single metal exposure (Sofo et al., 2013). When *A. thaliana*

seedlings were exposed to subtoxic concentrations of Cu, Cd and Zn alone and combined, the single exposures (and mostly Zn) caused an increase in average root diameter and total root length (combining primary root and lateral root lengths) due to an increase in the number of lateral roots and in average root diameter. Exposure to a mixture of the three metals, however, decreased these parameters, potentially due to the exceedance of non-toxic concentrations caused by a synergistic effect of the toxicants' combination (Bochicchio et al., 2015).

8 | CONCLUSIONS

Although this review is based on experiments using varying set-ups, it clearly shows certain toxicity patterns upon exposure of *A. thaliana* roots to Cu, Cd or Zn. Concerning the root system architecture, most prominently, all three metals affect primary root growth negatively, except for Cu and Cd having a beneficial effect at low concentrations. Only Zn was reported to cause abnormally branched root hairs. The response to toxic concentrations of these metals in a mixture as compared to individual applications seems to include the individual responses in a non-additive manner. On the other hand, at subtoxic concentrations of these metals (leading to no observable effect on root system architecture in single exposures), mixed exposure can lead to measurable toxicity. Important to note is that metals do not often have a negative effect on root growth after several hours, but already cause oxidative stress before this results in reduced biomass. The assessment of effects on root system architecture is most easily accessible on solid agar substrate, but in the context of an AOP this makes it difficult to relate these effects to effects on lower biological organization levels as these are mainly studied using hydroponics. In addition, different experimental setups can also affect a diverse outcome on mineral profiles and a large difference between the timing of the exposure, exposure length (Table S1), metal speciation and bioavailability complicates the comparison of studies, even though they use similar approaches.

Investigations of the cellular organization of roots treated with Cu, Cd and Zn show differences between the effect of these metals. The effect of Cu on primary root growth seems to be a consequence of disturbed mitotic activity, whereas Cd acts on cell differentiation mainly at the quiescent centre and Zn inhibits cellular elongation. This shows that the altered root system architecture of plants treated with these metals is achieved by effects on the cellular organization, which are unique for each metal. This is further supported by the different effects on hormonal homeostasis observed for each metal. There is an obvious difference in the mode of action of Cu, Cd and Zn based on their physico-chemical properties, affinities to functional groups and in the case of Cu and Zn their biological roles. This primarily affects the oxidative homeostasis and metal chelation and sequestration in roots leading to alterations in gene expression regulation.

From the above discussion, it becomes clear that to ameliorate comparison and extrapolation of results, standardized conditions for metal toxicity testing should be set. Nevertheless, the AOP framework (Figure 3) could help to integrate the available data that are present in the literature. In addition, further research should focus on exposure to concentrations that by themselves do not cause effects, but in multiple exposure conditions, as is often the case in contaminated soils, have a negative effect on the total growth of the plant.

CONFLICT OF INTEREST
The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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