



Hydroponics in physiological studies of trace element tolerance and accumulation in plants focussing on metallophytes and hyperaccumulator plants

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Abstract

Background When studying metallophytes and hyperaccumulator plants, it is often desired to assess the level of tolerance of a specific trace metal/metalloid in a putative tolerant species, to determine root and shoot accumulation of the trace metal/metalloid of interest, or to establish whether a trace metal/metalloid has an essential function. The use of hydroponics

has proven to be a powerful tool in answering such questions in relation to the physiological regulation of metal/metalloids in plants. Carefully designing experiments requires considering nutrient solution formulation, dose rate regime, and environmental conditions, but this is often overlooked.

Aims This review aims to bring together key information for hydroponics studies in physiological, evolutionary, and genetics/molecular biological research of trace metal/metalloid tolerance and accumulation in plants, focussing on metallophytes and hyperaccumulator plants.

Conclusions It is not possible to define a ‘universal’ nutrient solution that is both sufficient and non-toxic for all plants, although it is often possible, dependent on plant species under study and the research question to be addressed, to ‘adapt’ commonly used ‘standard formulations’. Well-designed and executed hydroponics experiments can yield powerful insights in the regulation of essential and toxic metal/metalloid trace elements, and this extends far beyond hyperaccumulator plants.

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Introduction

Trace metal and metalloid element hyperaccumulator plants have had much academic interest over the last four decades (Jaffré et al. 1976; Brooks 1998; Baker

and Brooks 1989; van der Ent et al. 2013; Jaffré et al. 2018). The total global inventory of hyperaccumulator plants stands at ~700 different species (Reeves et al. 2018, 2020) of which most (70%) hyperaccumulate nickel (Ni). A wide range of different elements from the periodic table can be hyperaccumulated, including cobalt (Co), copper (Cu), thallium (Tl), selenium (Se), cadmium (Cd), manganese (Mn) and zinc (Zn). Nominal threshold criteria have been established to distinguish hyperaccumulation, and hyperaccumulators are defined as plants that exceed in their shoot dry foliar matter $100 \mu\text{g g}^{-1}$ Cd, Se, or Tl, $300 \mu\text{g g}^{-1}$ Co or Cu, $1000 \mu\text{g g}^{-1}$ Ni or As, $3000 \mu\text{g g}^{-1}$ Zn or $10,000 \mu\text{g g}^{-1}$ Mn (van der Ent et al. 2013). Although exposure to a minimum trace element concentration in the growth medium is needed to achieve uptake, hyperaccumulator plants have highly efficient uptake and root-to-shoot translocation behaviour and show a non-linear response to substrate trace element concentrations (Baker 1981; Baker 1987). For example, *Noccaea caerulescens* can achieve $>20,000 \mu\text{g g}^{-1}$ foliar Zn when growing on soils with $<100 \mu\text{g g}^{-1}$ of this element (Reeves et al. 2001) or *Neptunia amplexicaulis* can achieve $>14,000 \mu\text{g g}^{-1}$ foliar Se when growing in (spiked) soils with $<30 \mu\text{g g}^{-1}$ of this element (Harvey et al. 2020).

Solution culture (hydroponics) is a powerful technique for untangling the levels of trace metal/metalloid tolerance and accumulation in hyperaccumulator plants, and finds numerous applications in physiological, evolutionary, and genetics/molecular biological research. Hydroponics culture is especially attractive because it permits one to singularly manipulate individual parameters, such as metal/metalloid exposure or ionic activity, pH, or ion interactions. Examples include competition between Zn and Cd in *N. caerulescens* (Zha et al. 2004), Zn inhibition of Cd accumulation in spinach (*Spinacea oleracea*) but not in rice (*Oryza sativa*) (Wang et al. 2023), or selective uptake of selenate over sulfate in *Astragalus bisulcatus* (Bell et al. 1992; Cabannes et al. 2011). Furthermore, hydroponics offers the opportunity to directly examine the roots, which is very difficult in soils, and hydroponics enables preclusion of extraneous contamination with dust particulates, especially important for studies focussing on lead (Pb) or chromium (Cr). This has important experimental benefits over studying much more complex systems, such as natural (or spiked) soils, at least in the first instance.

The chemistry of natural soils is immensely complicated which makes it hard (if not impossible) to control or know the exact metal/metalloid availability to the plant. The presence, absence, or unknown status (and the inability to control) rhizosphere bacteria and (mycorrhizal) fungi is another complicating factor in soil-based studies. However, hydroponics experiments involving putative hyperaccumulator plants have also been frequently misapplied to exceed tolerance limits leading to nonspecific ‘breakthrough’ of metal/metalloids into the shoot and spurious claims for supposed ‘hyperaccumulation’ (Baker and Whiting 2002). This situation applies specifically to many common weed species that are tested for their ‘capacity for hyperaccumulation’ by applying extremely high dose rates, while they are not known to exhibit hyperaccumulation in nature (van der Ent et al. 2015). In this way, almost any plant species can be made to ‘hyperaccumulate’ if dose-levels are sufficiently high, but this leads to severe stress and plant mortality. The problem thus lies in that hydroponics experiments often use unrealistically high dose treatments for single elements where the characteristic differences between hyperaccumulator and non-accumulator species tend to disappear due to saturation of the root-to-shoot translocation in the hyperaccumulator, or of the root’s sequestration capacity in the non-accumulator (van der Ent et al. 2013, 2015).

Virtually any plant species can be cultured in hydroponic and our laboratories have successfully grown temperate herbaceous species, such as *N. caerulescens*, *Arabidopsis halleri*, as well as semi-arid (desert) species, such as *Astragalus bisulcatus*, *Neptunia amplexicaulis*, and tropical woody rainforest species, such as *Macadamia integrifolia* and *Pycnanandra acuminata* (Fig. 1) (Abubakari et al. 2022; Isnard et al. 2020). Plants can be grown from seed (most herbaceous plants, typically first germinated on perlite/vermiculite mix and then transferred to hydroponics), or from cuttings (most woody plants, first rooted using hormone gel on perlite/vermiculite mix and then transferred to hydroponics). It is preferable to transfer young plants at the seedling stage (as early as possible) to the hydroponics experiment, rather than growing a fairly mature plant in soil or other medium, and then transfer to hydroponics to minimise ‘transplant shock’ and to ensure survival. We have developed a method in which seeds are sterilised, then grown in sterile Gelzan-based gel in small

Fig. 1 The tropical woody Ni hyperaccumulator *Pycnanandra acuminata* from New Caledonia grown in hydroponics (image from Isnard et al. 2020), top panel, and the tropical woody Mn hyperaccumulator *Macadamia integrifolia* from Australia also grown in hydroponics



2 mL Eppendorf tubes in which the bottom is cut-off. The tube with seedling can then be transferred directly into the hydroponics where the roots will grow and protrude out of the gel and into the hydroponics solution. This works particularly well for very small-seeded plants, such as *Arabidopsis* or *Noccaea*.

Soil is a highly complex, heterogenous system that varies in its properties with depth, varies across the landscape, and varies across time. Furthermore, experimental control and manipulation of soil is difficult due to the presence of a solid-phase which buffers the soil solution – experimentally changing one property of the soil often causes inadvertent changes to other properties thereby

confounding experimental datasets. In this regard, the use of solution culture (hydroponics) is a useful approach to avoid the experimental difficulties of soil given that nutrient solutions are more controllable due to the absence of a solid phase. However, where nutrient solutions are used in experiments as a substitute for the soil, care must be taken to ensure that the nutrient solutions in which the plants are growing does not mask or otherwise interfere with the treatment effects to be measured. This is of critical importance given that the composition of the nutrient solution can have a profound impact on the experimental results obtained. For example, use of irrelevant pH values, addition

of the element of interest at rates orders of magnitude too high, or the addition of background nutrients at rates that greatly exceed those found in soil solutions can all have substantial impacts on plant performance and hence the overall experimental findings. Thus, it is critical to be aware that plant behaviour can be altered by the composition of the solution it is growing in.

This review focuses specifically on aspects relevant to studies on trace metal/metalloid tolerance and accumulation in plants, focussing on metallophytes and hyperaccumulator plants, and we refer to the seminal reviews of Asher and Edwards (1983) and Parker and Norvell (1999) for a much more detailed analysis of the use of hydroponics in plant (nutrition) research. The aim of this practical review is hence to assist researchers in carefully designing experiments on the basis of nutrient solution formulation, dose rate regime, and environmental conditions. Well-designed and executed hydroponics experiments can yield powerful insights in the regulation of essential and toxic trace elements, and we hope that this discussion will stimulate to critically examine experimental design parameters.

Nutrient solution formulations

Why conduct hydroponics experiments?

The reasons for conducting hydroponics dosing experiments on metallophytes and hyperaccumulator plants, and therefore the design parameters, are manifold. These include studies to (i) assess the level of tolerance of a specific trace metal(loid) in a putative tolerant species, such as a plant found growing on metalliferous or contaminated soils; (ii) determine root and shoot accumulation of the trace metal(loid) of interest in a species that is ostensibly a (hyper)accumulator based on field data; and (iii) establish whether a specific trace metal(loid) has an essential function and to show deficiency of that element (Table 1). Hydroponics experiments can be conducted for addressing all of these aims whilst generating data on growth parameters and samples that can be used for physiological, biochemical, and genetics/molecular biological analyses.

Table 1 Main aims and methods for the range of studies that can be undertaken on metallophytes and hyperaccumulator species

Aim of experiment	Methodology	Analyses	Example studies
Assess level of tolerance to a trace metal/metalloid in a putative tolerant species	Use seeds of contrasting species or accessions, as tolerance is always comparative. Grow plants from seed to mature stage. Use sensible dose concentrations.	Root elongation test, root and shoot biomass, physiological stress indicators (biochemical, photosynthesis).	<i>Crassula helmsii</i> (Küpper et al. 2009), <i>Stellaria media</i> (Salinitro et al. 2020)
Determine root/shoot accumulation of a trace metal/metalloid in a (hyper) accumulator species		Elemental analysis (ICP-AES/MS, XRF), physiological stress indicators (biochemical, photosynthesis), biomass, elemental distribution (synchrotron or lab-based μ XRF)	<i>N. caeruleascens</i> (Brown et al. 1995; Kozhevnikova et al. 2020), <i>Pycnantha acuminata</i> (Isnard et al. 2020)
Establish essentiality of a trace metal/metalloid and show deficiency symptoms.	Use target element depleted seed stock. Cultivate plants in full lifecycle.	Elemental analysis (ICP-AES/MS, XRF), visual symptoms, biomass.	<i>Carya illinoensis</i> (Wood et al. 2004), <i>O. chalcidica</i> (Chaney et al. 2009)

What hydroponic solution formulation do I choose?

In any hydroponics experiment, it is key that the composition of the ‘background’ nutrient solution, which usually functions as the ‘control’ in most trace metal tolerance/accumulation experiments, is non-toxic and sufficient for maximum growth throughout the duration of the experimental treatments. In this regard, a plethora of nutrient solution formulations exists, but “*no one nutrient solution is superior to all other solutions*” (Hoagland and Arnon 1950). A key factor influencing the development of different nutrient solution formulations has been a focus on minimising nutrient depletion in the nutrient solution resulting from plant uptake of those nutrients, with uncontrolled depletion of these nutrients leading to severe deficiency and reduced growth. This comparatively rapid depletion of nutrients occurs due to the (deliberate) absence of the solid phase which would otherwise buffer the solution. As a result, very broadly, nutrient solution recipes can be divided into two groups, i.e. (1) “concentrated” nutrient solutions, in which the concentrations of all essential nutrients are far in excess of those required for maximum growth, and (2) “dilute nutrient solutions”, in which the nutrients concentrations are supplied at low concentrations, comparable to those in natural soil solutions.

Concentrated nutrient solutions contain nutrients (especially P) at values that generally exceed those found in soil solutions (Table 2.), with the aim being to supply an excess of nutrient so that its uptake does not cause deficiency phenomena, through depletion from the nutrient solution. Inadvertently reduced control growth, owing to deficiency or toxicity of the background nutrient solution within the duration of the experiment, is clearly undesirable.

The second broad type of nutrient solutions is dilute solutions that explicitly aim to mimic the chemical composition of soil solutions. Since there are often reasons to suppose that plant responses to any treatment may depend on experimental conditions, and thus not quantitatively reflect or predict their responses under ‘natural’ conditions, it can be rewarding, dependent on the underlying research question, to choose a background solution that mimics a natural soil solution. However, for such solutions, the uptake of nutrients (especially P) results in their rapid depletion in the nutrient solution (especially if the solution volume is relatively small, see [Nutrient solution formulations: dilute nutrient solutions](#) section), potentially causing nutrient deficiencies if appropriate care is not taken. Thus, knowingly, or unknowingly, when selecting a nutrient solution recipe, researchers are faced with a broad choice: select a nutrient solution with higher nutrient concentrations (being most pronounced for P) which will require less effort to maintain but will be less representative of the conditions experienced by plants in soils or select a nutrient solution with lower nutrient concentrations which will more accurately mimic soils but will be more difficult to maintain. These two broad types of nutrient solutions (concentrated and dilute) are discussed below in detail.

Nutrient solution formulations: concentrated nutrient solutions

Concentrated nutrient solutions, such as Hoagland’s, are often referred to as ‘classical’ recipes and are still extensively used. Concentrated solutions have the primary advantage of being less susceptible to plant-induced nutrient depletion (due to uptake), and

Table 2 Concentrations of selected nutrients in soil solutions compared to concentrations of nutrients in selected commonly used nutrient solutions

	Soil solution		Nutrient solution		
	Reisenauer (1966) ²	Barber (1995) ¹	Hoagland and Arnon (1950)	Brown et al. (1995)	Blamey et al. (2015)
NO ₃ -N (μM)	1500	-	14,000	5000	670
P (μM)	0.75	1.6	1000	100	5
K (μM)	1100	150	6000	2700	300
Ca (μM)	1700	1500	4000	2500	1000
Mg (μM)	3000	2500	2000	1000	95
S (μM)	450	-	2000	1000	340

¹Mode value of 134 values from Alfisols and Mollisols

²Median value for a total of between 979 soils (Ca) and 149 soils (P)

hence solution composition tends to be fairly constant over time, at least when solutions are replaced once or twice per week, and the root biomass per unit of solution is not excessive. Ideally, in concentrated solutions all basal nutrient concentrations should be far in excess of those required for optimum growth, but below their toxicity thresholds. The use of such concentrated solutions has two main disadvantages. The first is that the use of nutrient concentrations that are far in excess of those found in soil solutions may cause changes in plant behaviour, potentially altering the experimental findings obtained. The second is that the common practice of adding some nutrients at concentrations that are orders of magnitude higher than in soil solution can cause precipitation within the solution, with this problem being most pronounced for P. For example, when studying Pb accumulation and tolerance in various hyperaccumulating and non-hyperaccumulating metallophytes, Mohtadi et al. (2012) first grew plants in a basal solution containing 1000 μM P before transferring plants to solutions containing 0 μM P for a further 14 d, to avoid precipitation of insoluble Pb-phosphates in the nutrient solution. Another obvious risk of using concentrated nutrient solutions is toxicity. For example, the Cu concentration in the original Hoagland solution (1 μM) is almost certainly toxic, surpassing the highest No-Effect Concentration (NEC) for root growth for most plant species (Kopittke et al. 2018), at least for most Brassicaceae (Novello et al. 2020), which are all lacking functional HMA5;1, the vacuolar Cu transporter (Li et al. 2017). The fact that full-strength Hoagland's-based nutrient solutions may potentially be Cu-toxic has not been generally acknowledged thus far probably owing to the wide-spread use of FeEDTA as the Fe source (and this issue is discussed further below).

Nutrient solution formulations: dilute nutrient solutions

The second broad approach is to use dilute solutions for which nutrients are supplied and maintained at low concentrations. In general, dilute solutions may be expected to reduce the chance of unforeseen toxicity and reduce the chance of undesirable interactions of regular nutrients with the treatment effects. In this regard, it is useful to consider the composition of 'typical' soil solutions. Many studies have

provided data on soil solution composition, such as Wolt (1994), Barber (1995), and Reisenauer (1966), with large variation observed depending upon the soil type, management practices (such as fertilisation) and climate. Of particular interest, it is noteworthy that despite the comparatively high P requirements of plants (~0.3 wt% on a dry matter basis), soil solution P concentrations are very low and generally <5 μM (Table 2.). In soil, the solution P concentration is buffered strongly by the solid phase, from which P is released when solution P is depleted through plant uptake. As discussed later, this causes a problem in nutrient solutions (where solution concentrations are not buffered by a solid phase) where uptake of nutrients, especially P, can cause a rapid depletion in the nutrient solution when supplied at concentrations designed to mimic the low concentrations of soil solutions. As a result, dilute nutrient solutions need to be frequently replaced, or supplemented at least, to avoid depletion of the nutrients to limiting levels. Thus, compared to the use of concentrated solutions, this can be labour-intensive and requires more careful planning in order to avoid nutrients being depleted, inadvertently causing nutrient deficiencies. Another conceivable disadvantage is that dependent on the plant species under study, the minimally required nutrient concentrations are often unknown, or incompletely known at least, implying that the treatment effects may become less visible owing to deficiency phenomena, which is much less likely to occur in concentrated nutrient solutions.

Maintaining the nutrient solution composition

The ultimate aim in hydroponics metal/metalloid dosing experiment is to keep the concentration of the target element – as well as the overall composition – constant (e.g., not fluctuating due to depletion over the time of the experiment). There are several approaches that can be used to achieve this: (i) regular replacement of the nutrient solution volume, often once or twice a week for the duration of the experiment; (ii) increasing the volume of nutrient solution via flowing solution culture; (iii) programmed nutrient addition, whereby nutrients are added at varying rates to replace those that have been taken up by the plant, or (iv) use of buffers (such as resins or soluble chelators) to maintain selected nutrients at constant levels. (Degryse et al. 2006). The volume of the hydroponics

container and the rate of solution replacement should be based on the consumption of relevant ions in the solution. It follows that small, slow-growing plants can be cultured in smaller containers (<1 L), but large, fast-growing plants need substantially larger containers (>10 L) (Fig. 2). Many published studies use containers that are likely too small. A back-of-the-envelope calculation shows that a plant that has a yield of 5 g dry weight biomass per week containing 10 mg g⁻¹ K needs 50 mg K from the solution. Assuming the solution contains 2 mM K (=78.2 mg L⁻¹), the plant will have depleted most K from a 1 L container in 1 week. Resin-buffered systems can be used to provide a constant supply of ions in the solution (Checkai et al. 1987a, b; Checkai and Norvell 1992). It is preferable to use a sufficiently large volume such that the total nutrient (and dosed target element) supply exceeds the daily uptake at least 10-fold. Asher and Edwards

(1983) provided an equation to calculate how often solutions should be replaced to limit unwanted depletion of specific elements (either nutrients or dosed trace elements) which is adapted below:

$$F = \frac{D}{100} \cdot \frac{V}{W} \cdot \frac{C_i}{U}$$

where F is the required frequency of replacement (hrs), D is the tolerable degree of element depletion (%), V is the solution volume (L) per container, W is the fresh weight (g) of the plant, C_i is the initial element concentration (μM), and U is the expected nutrient uptake rate (μmol g FW⁻¹ h⁻¹).

Importance of solution pH control

In nutrient solutions, pH regulates speciation and solubility, and hence pH is often referred to as being the “master variable” (Rengel 2002). It follows that appropriate pH control is important because: (i) It should mimic the situation of interest; (ii) pH at extreme values, either too low or too high, is *directly* toxic to plants; and (iii) It influences speciation and solubility of elements, being particularly important for the element of interest. It follows that the pH of the nutrient solution is the single most important parameter for controlling ionic activities, as well as to prevent unwanted precipitation. Most often a solution pH of 5.8 is used, and this is compatible with many plant species and keeps key transition elements in solution. Vigorously growing plants have a dramatic effect on the solution pH (with up to 1 pH unit change per day) which need to be controlled by adding acid or base (typically HNO₃ or KOH) solution. More accurately, pH controllers with dosing pumps are now available to keep the solution pH stable (within 0.1 pH unit) for the duration of the experiment. The use of buffers can also help to keep the pH stable, and zwitterionic compounds, especially MES (2-(N-morpholino) ethanesulfonic acid) are suitable because it is non-toxic and not metal complexing. Finally, pH stabilisation can also be achieved by using a combination of ammonium N and nitrate N to balance H⁺ and HCO₃⁻ release from the roots upon N uptake.

The effect of pH on the relative activity of Ni²⁺ and complexes is shown in Fig. 3. It can be seen that Ni²⁺ free ion dominates up to pH 7.5, after which OH⁻ and CO₃²⁻ species take over. However, for



Fig. 2 The thallium hyperaccumulator *Biscutella laevigata* (top) and the selenium hyperaccumulator *Neptunia amplexicaulis* grown in hydroponics in 10 L plastic containers

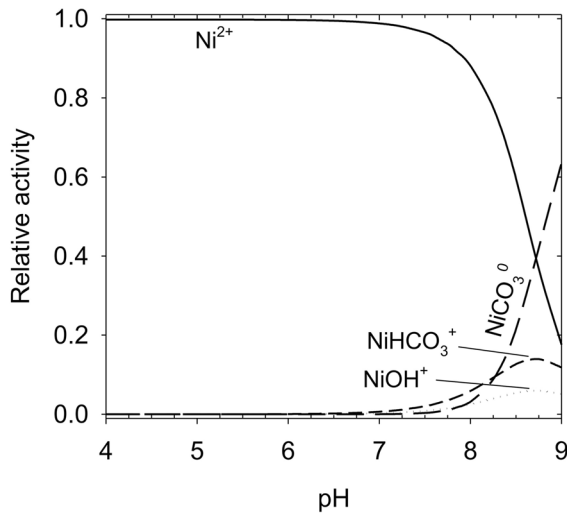


Fig. 3 Effect of pH on the relative activity of Ni^{2+} and complexes. Solutions were aerated with atmospheric CO_2 . Speciation was calculated using PhreeqcI v3.4 using the constants (from Gamsjäger et al. 2005) listed in Suppl. Table 1

transition elements, such as Co, Cu, Mn or Zn, the highest free ionic activity (within physiological relevant ranges) is pH 5.4–5.8 (although this is element specific and concentration dependent) and suitable for most hyperaccumulators of these elements. However, for Se hyperaccumulators that evolved on alkaline soils optimum selenate (SeO_4^{2-}) availability is at pH 7–8, whilst at lower pH and redox state selenite (SeO_3^{2-}) dominates (Mayland et al. 1991). Similarly, for As, the oxyanion (AsO_4^{3-}) is most available at pH 7–9, and therefore experiments involving Se and As hyperaccumulator plants should ideally be undertaken at neutral to alkaline pH.

Specific considerations for Fe-chelation interfering with other cations and phosphate

Although the supply of all nutrients is equally important, Fe is more difficult to supply appropriately. Unlike most nutrients, Fe must be supplied with a chelator to ensure that it remains soluble and plant-available. The supply of Fe without a chelator will result in its precipitation, leading to Fe deficiency. Unfortunately, keeping Fe available in solution through the use of a chelator without interfering with other transition metals (Cu, Ni, Mn, Zn) is not trivial. Key to providing adequate Fe and avoiding interferences with

transition metals is the use of specific chelators that have a higher stability constant for Fe^{3+} than for transition metals. This can be achieved by using either EDDHA (ethylenediamine-N,N'-bis(2-hydroxyphenyl)acetic acid) or HBED (N,N-bis(2-hydroxybenzyl)ethylenediamine-N,N'-diacetic acid) as the Fe chelating agent. The use of EDTA is common in hydroponics experiments but should be strictly avoided as Fe can be displaced by many transition metals, such as Cu, Ni and Zn from the FeEDTA complex. This will consequently lead to incorrect metal dose rates and Fe starvation in the plants. For example, Cu has a much higher affinity toward EDTA than Fe, at least within the commonly applied pH range for nutrient solutions (pH 4.5–6.5). If Cu is supplied at concentrations less or equal than Fe-EDTA, which is usually supplied at 20 μM , >98% of Cu will be complexed by EDTA. This results in detoxification of Cu, as Cu-EDTA is >100-fold less toxic than hydrated Cu^{2+} , and displacement of an equivalent amount of Fe from the EDTA complex (Fig. 4). Consequently, Fe is removed from the solution through precipitation, either as an oxo-hydroxide, or a phosphate, dependent on the solution pH and composition. In any case, this displacement reaction proceeds only slowly, reaching equilibrium not until one or more days, dependent on pH, meaning that freshly prepared Hoagland's solution, even when prepared with FeEDTA, will initially be Cu-toxic. Furthermore Zn, usually supplied at 2 μM in full-strength Hoagland's solution with FeEDTA, will finally become bound to EDTA, albeit less rapidly and less completely, but 2 μM Zn has not been shown to be toxic. Regarding trace metal tolerance studies, it is worthwhile to note that also Cd will become partly bound by EDTA in FeEDTA-containing nutrient solutions, albeit incompletely and only considerably at pH >5. Similarly, Pb will become EDTA-bound, at least as rapidly and completely as Cu. These are clearly instances of undesirable interactions of the treatment variable (a toxic metal) with a regular nutrient (FeEDTA). These interactions can be simply prevented by using a more specific Fe chelator, e.g., FeHBED (Chaney 1988). Because grasses accumulate Fe using phytosiderophores which chelate the Fe^{3+} , Fe-chelates used to supply Fe need to consider the ability of phytosiderophores to exchange Fe^{3+} from the added Fe-chelate. Experience has shown that it is possible to buffer all microelement cations using HEDTA and allow grasses to thrive.

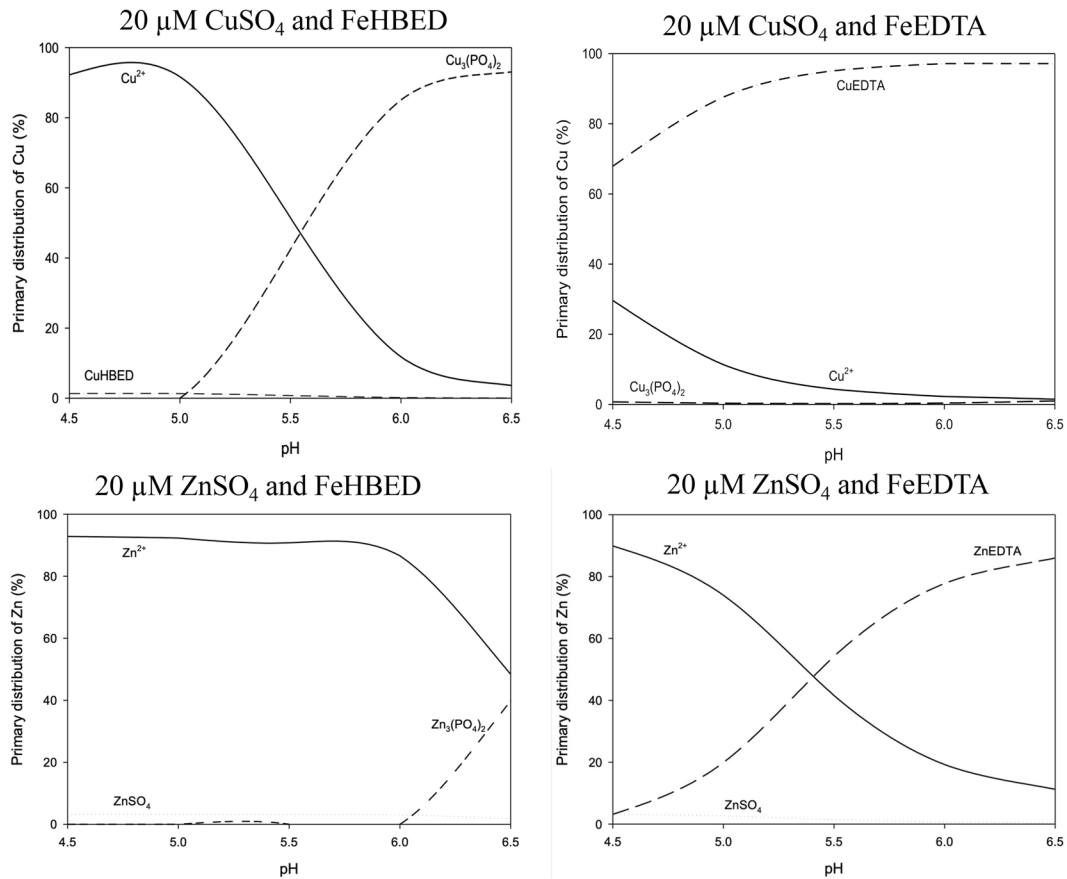


Fig. 4 Effect of pH on the speciation of Cu²⁺ and Zn²⁺ displacement with Fe-chelators at different pH values of the solution (½ Hoagland formulation) calculated with Geochem-EZ

Buffering microelement cations with DTPA or EDTA does not allow adequate Fe as the excess chelator, which is mostly CaEDTA, limits Fe phytoavailability through the phytosiderophore equilibrium. In general, when needed, e.g., in trace metal/metalloid accumulation experiments, low ion metal/metalloid ion concentrations in solution can be kept at desired and constant levels using resin- or chelator-buffered systems as mentioned previously.

Another element that can be troublesome in hydroponics is P due to its ability to precipitate both macro elements (such as Ca) and most transition elements, including essential nutrients (e.g., Fe, Zn). One can use hydrogen phosphate salts ([HPO₄]²⁻) mixed separately and last in the final solution to avoid precipitation or use a low concentration of phosphate (5 μM PO₄-P) in the solution and add small daily aliquots

software (values provided in Suppl. Table 2). Note: the phosphate (PO₄) salts are solid precipitates

during the experiment to replenish P (see for example, Blamey et al. 2015). For example, culturing *Macadamia species*, like most Proteaceae, requires low concentrations of P (< 100 μM).

Metal/metalloid chemical forms and dose rates

Typically, metal(loid)s are supplied in a form that is instantly and freely available to the plant for uptake by the roots. In the case of transition elements, such as Ni, Zn, Cd, either their sulphate or nitrate salts are typically used. In some cases, the valency may be important, for example Mn (2+ most commonly, but 3+ and 4+ are also present in biological systems), Co (2+ under normal conditions, but 3+ can exist under oxidizing conditions). The case of Cr is special because Cr is present as CrIII under standard

environmental conditions in soil, but has poor solubility, and in many studies CrVI is dosed in hydroponics. Although CrVI can exist in nature (in some soils CrIII is oxidised to CrVI by Mn-oxides), the relevance of using hexavalent Cr for physiological studies is highly questionable.

Other transition elements form oxyanions, including V (vanadate, VO_4^{3-}) and Mo (molybdate, MoO_4^{2-}) and are supplied in this form. The metalloids As and Se deserve separate mentioning as their valency is crucial for uptake in hyperaccumulator plants. *Pteris vittata* takes up both arsenate (AsV, AsO_4^{3-}) and arsenite (AsIII, AsO_3^{3-}) with different transport pathways, arsenite as undissociated H_3AsO_3 via aquaporins, arsenate via phosphate-transporter pathways (DiTusa et al. 2016). Selenium hyperaccumulators, such as *Astragalus bisulcatus*, take up selenite (SeIV, SeO_3^{2-}) via phosphate transporter pathways and selenate (SeVI, SeO_4^{2-}) via sulfate transporters (White 2018). It may sometimes be desirable to use slow-release forms of the target elements to provide a continuous supply and/or to prevent (acute) toxicity. This may be achieved by using a salt that easily dissociates, for example zinc carbonate (ZnCO_3) readily weathers and releases Zn^{2+} . Loaded ion-exchange resins may also be used to affect a constant supply of the target ion in solution (Checkai et al. 1987a, b; Checkai and Norvell 1992).

Testing for Cd (hyper)accumulation deserves a special caution in relation to Zn status. All known Cd hyperaccumulators (such as *N. caerulea* or *Sedum plumbizincicola*) are a subset of Zn hyperaccumulators and the soils on which they occur in nature usually has at least 100-fold more Zn than Cd (Baker and Brooks 1989; Reeves et al. 2020). Therefore, testing a target species by solely supplying it with 10 μM Cd without the necessary 1000 μM Zn gives the false impression that it is a genuine Cd hyperaccumulator capable of high Cd uptake in the presence of Zn. This is crucially important for any practical applications in phytoremediation where Zn is almost always greatly in excess of Cd in either geogenically-enriched soils or in contaminated soils.

The dose rates of any experiment should be realistic with expected physiological responses. Any search in scientific databases will reveal numerous examples in the literature of extremely high-level dosing of a metal/metalloid leading to supposed 'hyperaccumulation' in the studied species. In order to help

to distinguish genuine hyperaccumulators, exposure levels must be kept low (e.g., $<5 \mu\text{M}$ for Ni, Zn, Cd, Tl). Another matter pertains to the hormesis effect, which is a biphasic dose response where a beneficial (growth promoting) effect is often observed when a potentially toxic element is dosed at a very low dose rate (Poschenrieder et al. 2013). For example, many plant species show a positive growth promoting response when dosed with 1 μM Se, even though this element is not essential to plants (Schiavon and Pilon-Smits 2016). There can be genuine reasons for high-level exposure treatments to demonstrate exceptional levels of tolerance in the target species, but this should always be accompanied with adequate assessments of plant health (for example, through measurement of root elongation, growth rate, chlorophyll activity, etc.). An example is a hydroponics experiment in which *Pycnantha acuminata* was exposed to 3000 μM Ni to demonstrate extreme tolerance in this species, which resulted in only a minor reduction in growth and no obvious toxic symptoms (Isnard et al. 2020). In fact, dosing at 100 μM Ni (a concentration lethal to virtually all other plants) was beneficial to growth in *P. acuminata*.

Comparisons between plant species-pairs

When using species-pair comparisons, e.g., those aimed at comparing 'tolerance capacities', it is often preferable to expose the hyperaccumulator model and its non-hypertolerant reference to different, but equally toxic concentrations, rather than equimolar concentrations (Schat and Kalff 1992). Generally speaking, many studies, aimed at comparing transcript or metabolite levels, or trace metal(loid) accumulation between hyperaccumulator and non-hyperaccumulator or non-hypertolerant reference species/populations, are easily biased by unrealistic dose levels, often exceeding the EC_{100} (i.e., the lowest 100%-effect concentration for root elongation) for the non-hypertolerant reference. For example, as shown by Schat and Kalff (1992), various Cu-hypertolerant populations and a non-metallicolous, non-hypertolerant population of *Silene vulgaris*, accumulated approximately the same amounts of phytochelatins (PCs) in their roots, when each population was exposed to its own EC_{50} for root elongation i.e., the concentration that inhibits root elongation by 50%. However, at exposure levels exceeding the

EC₁₀₀ of the reference population, the Cu-hypertolerant populations synthesised more PCs than the reference population (of which the roots had started to die off), while the opposite (higher PCs accumulation in the reference population) was apparent at exposure levels lower than the EC₅₀ (of the reference population). This example clearly demonstrates that dosage rates should be chosen on the basis of a detailed knowledge of the dose-effect relationships of the species or populations to be compared, to avoid any confusion of potential determinants of (hyper)tolerance or (hyper)accumulation with ‘toxicity symptoms’. If such knowledge is not available, then it is often better to keep the exposure level as low as possible, or if reasonably possible, to apply a broad series of exposure levels, to enable comparisons at comparable toxicity levels afterwards (Novello et al. 2020). Figure 5 shows examples of typical growth and root elongation

responses to dosing with metals in hyperaccumulator and non-accumulators or contrasting accessions.

Experimental duration

In general, the preferable duration of an experiment depends on the research question addressed. The duration of the hydroponics experiment is not often considered. Numerous examples exist in the literature about inordinately short exposure to the dosed element(s) being studied. In most cases plants were grown in hydroponics without the dosed elements and transferred to a treatment with the elements for just a few days. This is clearly much too short to reach metabolic homeostasis. Unless time-resolved uptake/translocation processes are being studied (e.g., ‘pulse-chase’ type of experiments), it is strongly preferable to grow a plant from seedling stage until mature

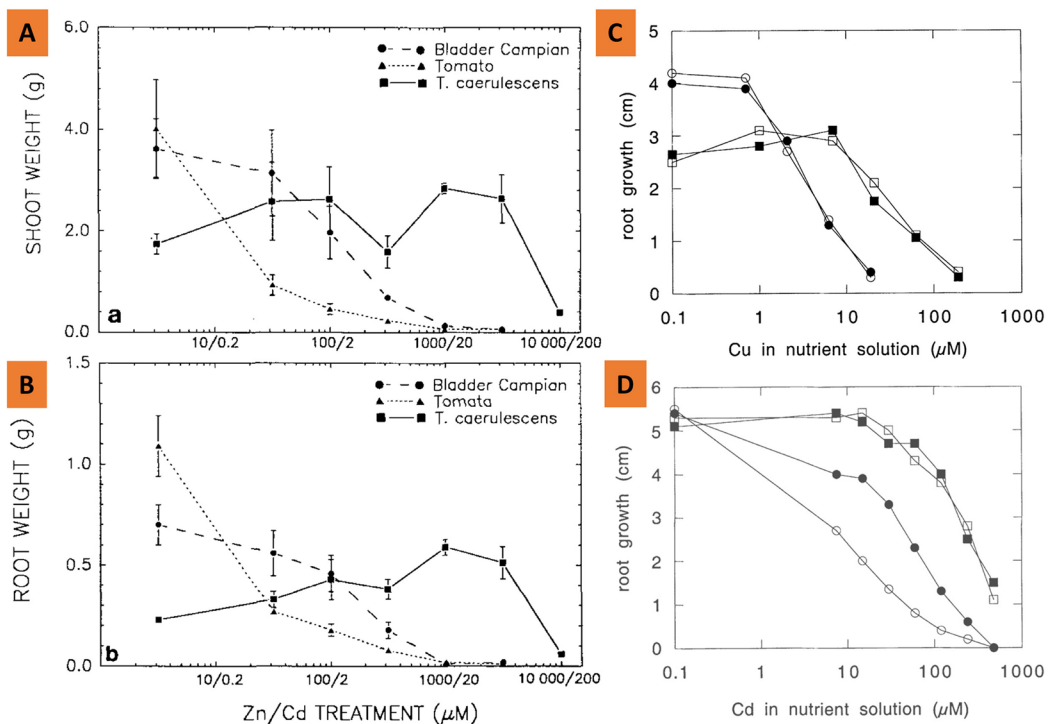


Fig. 5 Typical growth (shoot and root weight) and root elongation responses to dosing with metals (in this case Zn, Cd and Cu) in hyperaccumulator and non-accumulators or contrasting accessions. Left panels are from Brown et al. (1995) and show biomass of (a) shoots and (b) roots of *Nocca caeruleus* (hyperaccumulator), *S. vulgaris* (indicator), and *Lycopersicon lycopersicum* (susceptible species) grown in hydroponics with Zn added at a 50:1 ratio to Cd across seven Zn/Cd treatments.

Right panels are from Schat et al. (2002) and show mean root elongation ($n = 15$) throughout 4 d of exposure to Cu in BSO-treated (L-buthionine-[S,R]-sulphoximine, BSO, an inhibitor of PC synthesis) (open symbols) and BSO-untreated (closed symbols) non-metallicolous (circles) and cupricolous (squares) *Silene vulgaris* (c) and exposure to Cd in BSO-treated (open symbols) and BSO-untreated (closed symbols) non-metallicolous (circles) and Cd-hypertolerant (squares) *S. vulgaris* (d)

under the treatment conditions. In the case of *N. caerulea* this equates to about 4 weeks and for *Odontarrhena chalcidica* this typically takes 6–8 weeks, but for many other plants may be longer. Alternatively, in tolerance tests, using root elongation as an end point, a few days is sufficient, particularly when the roots are stained at the start of exposure (e.g., Schat et al. 1993). Although root responses may still increase after 2 days, and shoot responses may not yet be visible then, short-term root growth tests (1–4 days) are sufficient to reflect even subtle genetic differences in tolerance. Also, when assessing prevailing metal(loid) uptake capacity levels, for example, to establish their Michaelis-Menten kinetics (K_m and V_{max}), experiments up to 24 h are key to prevent significant changes in biomass, and avoid transcriptional or posttranscriptional change of the responsible transporter activities. In such short-term experiments, the risk of interference through deficiency phenomena is negligible, and therefore, a complete nutrient solution background solution will not be required. For example, measuring Cu tolerance in a 4-day root growth test produced identical results in a background solution with and without FeEDDHA (H. Schat, unpublished).

Many hyperaccumulator plant species have seeds that contain very high concentrations of the hyperaccumulated element. This can make studies aiming to determine a physiological requirement for certain trace elements that are also hyperaccumulated rather difficult. For example, *N. caerulea* seeds can contain $>700 \mu\text{g g}^{-1}$ Cd (van der Ent et al. 2022) and *Odontarrhena muralis* (actually *O. chalcidica*) seeds can contain $>10,000 \mu\text{g g}^{-1}$ Ni (Paul et al. 2020). In those cases, it may be necessary to grow mature plants in hydroponics with very low concentrations of the hyperaccumulated element to generate seeds with low concentrations of the element in question. Chaney et al. (2009) attempted to induce Ni deficiency in *O. corsica*, but this was unsuccessful due to high Ni concentrations in the seed, but plants on the lowest Ni activity reached $1\text{--}2 \text{ mg Ni kg}^{-1}$ and remained healthy (Chaney et al. 2009). Similarly, if testing for deficiencies of essential micronutrients, especially for which requirement is very low (for example, Ni as part of the enzyme urease in ‘normal’ plants), then it may be necessary to grow successive generations under limiting conditions to obtain depleted seed stock. This is extremely challenging for

normal plants that have a very low Ni requirement, for example oat (*Avena sativa*), to be able to induce Ni deficiency (Brown et al. 1987a, b).

If all conditions (solution chemistry, temperature, light) are optimised for the specific species, then hydroponics can offer highly enhanced growth rates compared to culture in soil. For example, the Se hyperaccumulator *N. amplexicaulis* reaches the mature flowering stage in <6 weeks in hydroponics whereas in soil this takes >12 weeks (Harvey et al. 2020). This is particularly advantageous undertaking more experiments or testing more species/accessions in the same time span. This can be further accelerated by using so-called ‘speed-breeding’ approaches which entail growing plants under increased lighting regimes with 22–24 h light per day (Watson et al. 2018; Ghosh et al. 2018). This can be put to use in phenotyping of plant traits, mutant studies, transformation and making crossing or selfed lines.

Chemical modelling of ion activities in solution

The use of chemical modelling software permits to predict specific ion activities in the solution and occurrence of unwanted precipitates and/or unwanted interactions of the ions in solution. This is highly recommended to assess before setting up any experiment, and whilst we do not discuss this detail here, we refer to Parker et al. (1995) for an in-depth treatise of dealing with the technical aspects of chemical modelling. The software package Geochem-EZ has been most frequently used for hydroponics solutions (Shaff et al. 2010). It is always advisable to run the final hydroponics solution formulation, inclusive of elemental dose treatments, through Geochem-EZ software to ensure the solution has the desired properties. Using such programs allows one to prepare chelator-buffered nutrient solutions to achieve the desired chemical activity of microelement cations similar to soil solutions, and to induce deficiencies in highly efficient species. Parker (1997) illustrated the use of HEDTA to induce Zn deficiency in six species. Chaney et al. (1992) used DTPA buffered nutrient solutions to provide varied severity of Fe stress to screen for chlorosis resistance in soybean.

Chelator-buffered solutions allowed identification of the very high Zn activity required by *N. caerulea* (Li et al. 1995). When they tried to grow this hyperaccumulator in nutrient solutions with Zn

activity buffered at Zn^{2+} levels adequate for many other species ($pZn=10$), *N. caerulescens* simply did not grow (Li et al. 1995). An experiment was conducted using a weaker Zn chelator (EGTA) to allow growth of *N. caerulescens* at the higher Zn^{2+} activity requirement compared with *T. arvense* and *Brassica oleracea* (Fig. 6). The adaptation of *N. caerulescens* to high Zn soils included change in Zn uptake transporters so it can tolerate and require 10^4 higher Zn^{2+} activity for this species to grow (Li et al. 1995). *Odontarrhena chalcidica*, *B. coddii* and *B. oleracea* were grown with soluble Ni and pH buffered across the range of 5.8 to 7.5 using MES and HEPES buffers (Fig. 7). Activity of Ni^{2+} did not change with pH, but Ni accumulation by both *Noccaea* and *Berkheya* had increased as pH rose, while Ni in cabbage declined with increasing pH. Using FeHBED assured adequate Fe supply without affecting Ni^{2+} activity across the pH range tested (Peters 2000).

Environmental conditions

Temperature and lighting conditions

Temperature regimes should be fitting to the species being studied, that is cooler (20–24 °C) for temperate species and warmer (28–32 °C) for tropical species.

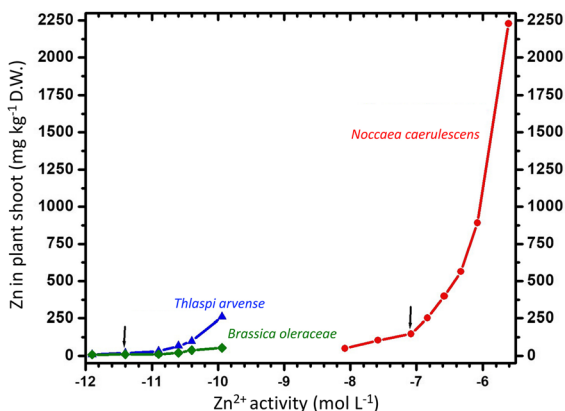


Fig. 6 Effect of chelator-buffered Zn^{2+} activity on Zn concentration in shoots of *Noccaea caerulescens*, *Thlaspi arvense* and *Brassica oleracea*. Arrows mark the activity where Zn^{2+} began to be adequate for normal growth of these species (10^4 -fold higher for *N. caerulescens*) (figure created based on data reported in Li et al. 1995)

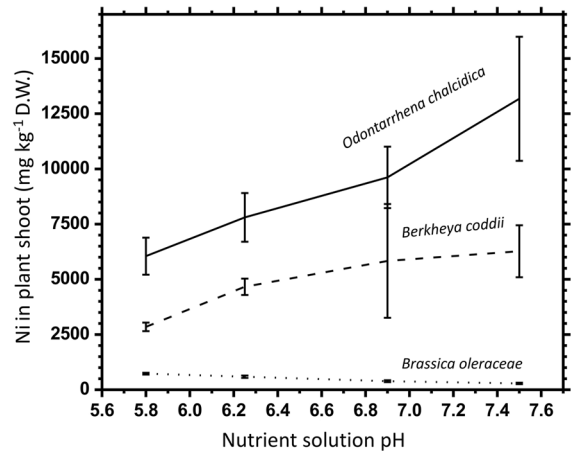


Fig. 7 Effect of nutrient solution pH (buffered with MES or HEPES) on Ni accumulation in shoots of *Odontarrhena chalcidica*, *Berkheya coddii* and *Brassica oleracea* (figure created based on data reported in Peters 2000). Plants were grown in 0.5 strength Hoagland solution, modified to mimic the concentrations of ultramafic soils (1 mM $MgSO_4$, 1.0 mM $Mg(NO_3)_2$, 1.5 mM $Ca(NO_3)_2$, 2.5 mM KNO_3 , 0.1 mM K_2HPO_4 , 1.0 mM NH_4NO_3 , 100 μM HCl, 15 μM H_3BO_3 ; 2 μM $MnCl_2$, 0.5 μM $CuSO_4$, 0.2 μM $NaMoSO_4$, 1 μM $ZnSO_4$, 20 μM FeHBED). Solutions were made in 4 replications in 8 L buckets and Ni was supplied as $NiSO_4$ at two treatment strengths (31.6 μM and 316 μM)

This should be optimised for maximising growth. Many temperate climate species need cold stratification for dormancy-breaking for germination and this can even differ between populations of species (Milberg and Andersson 1998). Usually placing seeds in a fridge at 3 °C for two to four weeks is sufficient. This also helps in obtaining uniform germination so that equal sized plants can be used for the experiment. If flowering is desired, then vernalisation can be required by temperate species, especially Brassicaceae, for example placing mature *B. laevigata* plants in hydroponics containers at 3–8 °C for two weeks promptly induces flowering, although *N. caerulescens* needs at least six weeks vernalisation.

One of the most important, and frequently overlooked aspects, is the quality and quantity of the light provided to the plants in the hydroponics culture. In numerous studies, inferior light sources are used, such as low-intensity ($<200 \mu mol m^{-2} s^{-1}$) fluorescent tubes with poor spectral quality for plant growth. Daylight in Summer in temperate regions has a photosynthetic photon flux density (PPFD) of $1200\text{--}1500 \mu mol m^{-2} s^{-1}$ and in tropical regions this

can be up to $2200 \mu\text{mol m}^{-2} \text{s}^{-1}$. The advent of high-intensity ($> 1000 \mu\text{mol m}^{-2} \text{s}^{-1}$) LED lighting that has photosynthetically active radiation (PAR)-light (400–700 nm) spectra or full-spectrum (350–1100 nm) lights that mimic natural solar radiation have revolutionised experimentation in the plant sciences. LED-based light sources can not only provide light of the optimum spectral quality but are much more efficient (about 40–50%) compared to high-pressure sodium or sodium halide lighting. Most plants need $> 500 \mu\text{mol m}^{-2} \text{s}^{-1}$ for maximum chlorophyll activity for optimum growth (Fig. 8), whereas some plants (especially plants from semi-arid regions) can even use up $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$ or higher. Moreover, C4 plants (including many grasses, as well as dicotyledonous species), for example some Amaranthaceae, are more efficient than C3 plants (Percy and Ehleringer 1984). Again, the overall aim is to enhance the visibility of the treatment effects, which in almost all cases means maximising growth throughout the duration of the experimental treatments. Therefore, we recommend determining the species-specific photosynthesis rates in response to light intensity ('light response curve') as values differ greatly between species (Zhu et al. 2010). Then use a light intensity in the experimental setup close to the saturation for the species in question to maximise growth.

There are only a few instances in which the quantity of light has a direct effect on the toxicity of the element dosed, notably in the case of Mn. Solar radiation is a potent trigger for Mn toxicity and leads to chlorophyll destruction and/or photobleaching in many plant species under Mn stress (Fernando and Lynch 2015). In contrast, deficiency symptoms have been reported for Mg to depend on light conditions, with high light intensity resulting in a higher requirement for Mg (Cakmak and Yazici 2010).

The quality (i.e., the composition of the spectrum) is often ignored but is critically important. Plants grown under identical conditions with the same photosynthetic photon flux density, but different spectral composition, can look totally different. Blue light (400–500 nm) is important for stomatal opening and chlorophyll synthesis, whereas red (600–700 nm)/far-red (700–750 nm) ratio determines elongation in plants. Finally, circadian rhythms in plants are also influenced by light quality (McClung 2001).

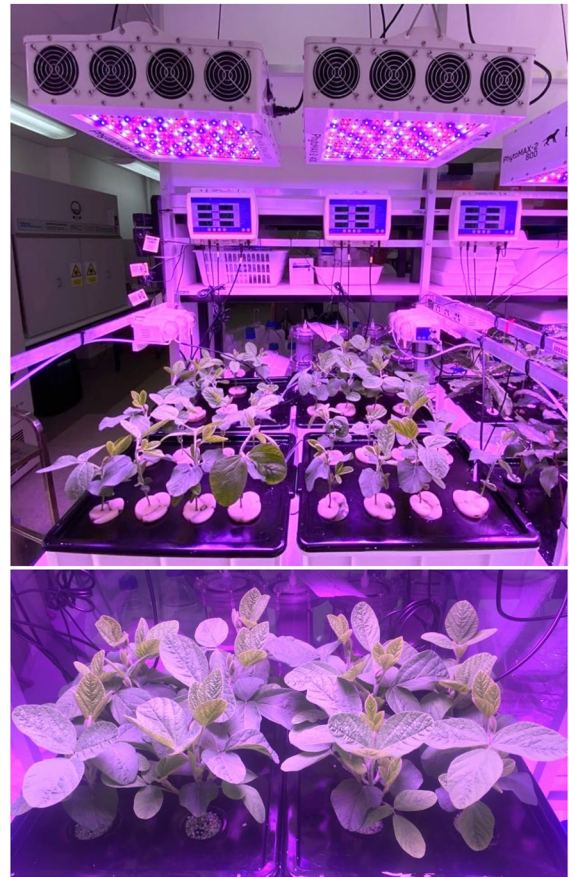


Fig. 8 Soybean (*Glycine max*) grown in hydroponics under Ni limiting conditions. The small plants are grown in 10 L plastic containers under high-intensity ($1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR light) LED lighting. Plastic covers are black to avoid algae growth in the containers

Aeration and aeroponics

Adequate aeration is often neglected, and many studies use no aeration of the solution at all with apparently successful experimental outcomes. The experience of our groups shows that aeration results in better plant growth. Typically, simple aquarium air pumps with air stones are used to provide aeration. This has the additional benefit of constantly mixing the solution to avoid 'dead' zones. Better still is to use high pressure (1–2 bar) pumps with ceramic air stones which provide much finer bubbles and hence better oxygenation. High levels of oxidation are also achieved in so-called ebb-and-flow hydroponics system, but this will not be discussed further as it has

no specific advantages over standard hydroponics. The oxygen saturation of the solution is also directly responsible for the redox state (ORP) of the solution and therefore potentially affects the chemical form of some elements that are dosed, for example it could lead to oxidation of arsenite to arsenate.

Doing away with a solution altogether, aeroponics relies on growing plants with roots in a mist environment. Although the concept is fairly well-known, there is a paucity of studies in the plant sciences that have used aeroponics. Plants are free-hanging in plastic ‘hydroponics’ containers and mist jets spray very fine (< 100 μm size) droplets of nutrient solution in this space. It requires high-pressure pumps (10 bar) and specialised tubing and misters. Droplet size is crucial and should be as small as possible so that the mist is persistent and to avoid fully wetting the roots. Alternatively, ultrasonic transducers can be used to generate even finer droplets (1–5 μm). Benefits of the aeroponics approach include much faster and more vigorous growth rates (up to double compared to hydroponics in our experience, Fig. 9) and formation of more ‘natural’ root systems that have root hairs. Typically, the aeroponics system runs ‘drain-to-waste’ which means the mist will have a constant composition, which is advantageous in metal(loid) dosing experiments. Disadvantages of this approach is the complexity of the equipment involved and the propensity for misters to clog due to bacterial growth.

Algae and micro-organism control

We recommend using black/dark lids on the hydroponics boxes to keep light reaching the solution to an absolute minimum to avoid algae growth. Nevertheless, algae tend to grow on the sponges for holding the plants. Instead, polypropylene beads can be used, but these are not suitable for (very) small plants. Algae growth is often only an issue in the “control” and not in the dosed treatments due to toxicity of trace metal(oids), especially Cu^{2+} . Unwanted micro-organism growth is not typically a problem in regularly replaced hydroponics, but can present a major problem in recirculating systems, and in aeroponics where even minor growth can block the small apertures of the misting jet nozzles. The use of in-line filters, or antibiotics (for example, the use of Rifampicin to control bacterial growth, see Phillips et al. 1981), may then become necessary. Another reason to



Fig. 9 Sunflower (*Helianthus annuus*) grown in high-pressure aeroponics achieving seed to flower in 10 weeks. The system comprises of high-pressure (10 bar) water pumps connected with high-pressure tubing the misting jets that spray a fine mist in the large plastic containers. Note ‘natural’ root system with abundant fine root hairs

prevent light exposure of the solutions is the photodecomposition of some Fe-chelates with FeEDTA being especially susceptible, although this can be limited by using a filter to remove the UV part of the spectrum (Yi and Guerinot 1996; Albano and Miller 2001).

Plant analysis

Chemical analysis of excised tissue samples

Ultimately, the purpose of a hydroponics experiment is to make observations and to generate samples that may be used to determine dose-response effects on growth, to examine the interactions between the (hyperaccumulated) element(s) of interest and other

plant nutrients, etc. Therefore, at the conclusion of the experiment, or at intervals during the experiment, samples will be collected for elemental analysis and/or to determine metabolites or other compounds. Care needs to be taken to select appropriate samples for analysis if not the whole plant is to be analysed. There will be major differences between the ionome/metal-ome of young apical leaves and old basal leaves, and between young growing roots and the main root stock. For many elements, older tissues contain (much) higher concentrations than younger tissues, especially of elements such as Ca and Si. Improvements in inductively coupled plasma atomic emission spectroscopy (ICP-AES)/mass spectrometry (ICP-MS) and acid digestion protocols enable to use exceedingly small amounts of samples. It is now possible to use a micro-scaled microwave digestion procedure for plant samples (1–20 mg d.w.) to obtain accurate elemental profiling (Hansen et al. 2009). This is useful for analysis of excised samples during the course of the experiment or for analysis of seedlings or very small plants (such as *Arabidopsis* tissues).

Non-destructive elemental analysis of plants

It is often desirable to obtain information on trace metal(loid) dose-rate induced toxicity (or conversely tolerance) effects during the hydroponics experiments. Even though small samples could be excised and analysed destructively, as described above, non-destructive methods can provide real-time information. Observation of characteristic visible symptoms can provide a wealth of information about trace metal(loid) deficiency/toxicity. For example, cellular-level Fe deficiency induced by Cd toxicity typically results in interveinal chlorosis (Wong et al. 1984), Ni-deficiency manifests itself as mottling of the young growth called ‘mouse-ear disease’ (Wood et al. 2004), and Mn toxicity can reveal itself by the formation of brown necrotic lesions on the leaves (Horst 1988). Although gross biomass production provides a crude assessment of the overall health of a plant, there are also more direct methods to probe the functioning of a plant. The use of chlorophyll fluorescence is a direct non-destructive method that probes photosynthesis system which is supremely sensitive to the effects of metal(loid) deficiency/toxicity (for applications in this context refer to Küpper et al. 2007; 2009). The advent of more sensitive handheld X-ray fluorescence

(XRF) devices has even made it possible to measure elemental concentrations in plant leaves non-destructively during the experiment at time intervals to look at kinetics of uptake and accumulation (van der Ent et al. 2019). Examples of synchrotron μ XRF elemental maps of a Se hyperaccumulator are shown in Fig. 10.

Elucidating the spatial distribution and prevailing concentrations of elements is a powerful approach to glean the physiological functioning of a plant, enable comparisons between plant species or accessions from dose-treatments (van der Ent et al. 2018; Kopittke et al. 2018; 2020). Synchrotron and laboratory-based (μ XRF) provide a level of sensitivity for full characterisation of the range of elements in plant tissues and is the only approach for determining the coordination environment of metal/metalloid complexes in situ (van der Ent et al. 2018). The technical capabilities of μ -XRF have been rapidly improving, now even allowing for time-resolved (kinetic) in vivo analyses of metal(loid)s with low detection limits, excellent resolution, and no theoretical restrictions on sample size (Kopittke et al. 2018, 2020). For example, using synchrotron-based μ -XRF, the changes in the distribution of Mn (and other elements) in the leaves of cowpea (*Vigna unguiculata*) upon exposure to elevated concentrations of Mn in the rooting medium was assessed over a period of 48 h (Blamey et al. 2018). Similarly, laboratory-based μ -XRF was used to examine the effects of Si dosing on the Mn status of soybean (*Glycine max*) and sunflower (*Helianthus annuus*) grown at elevated Mn in solution in a time-series (van der Ent et al. 2020). The obvious benefit of a laboratory μ -XRF instrument is that it will be generally available when experimental needs dictate readiness when undertaking trace metal(loid) dosing trials on plants (Mijovilovich et al. 2020), as access to synchrotron facilities is typically *via* a competitive beamtime application process. Compared to synchrotron facilities, laboratory μ -XRF has a lower X-ray flux, resulting in longer scan times, and inability to conduct X-ray absorption spectroscopy (XAS) to determine chemical speciation of the target element (Kopittke et al. 2018; van der Ent et al. 2018). Finally, the possibility of radiation-induced damage in μ -XRF analysis (especially in fresh hydrated samples) is an important consideration that may limit the information sought from the analysis (van der Ent et al. 2018). In a recent study radiation dose limits for

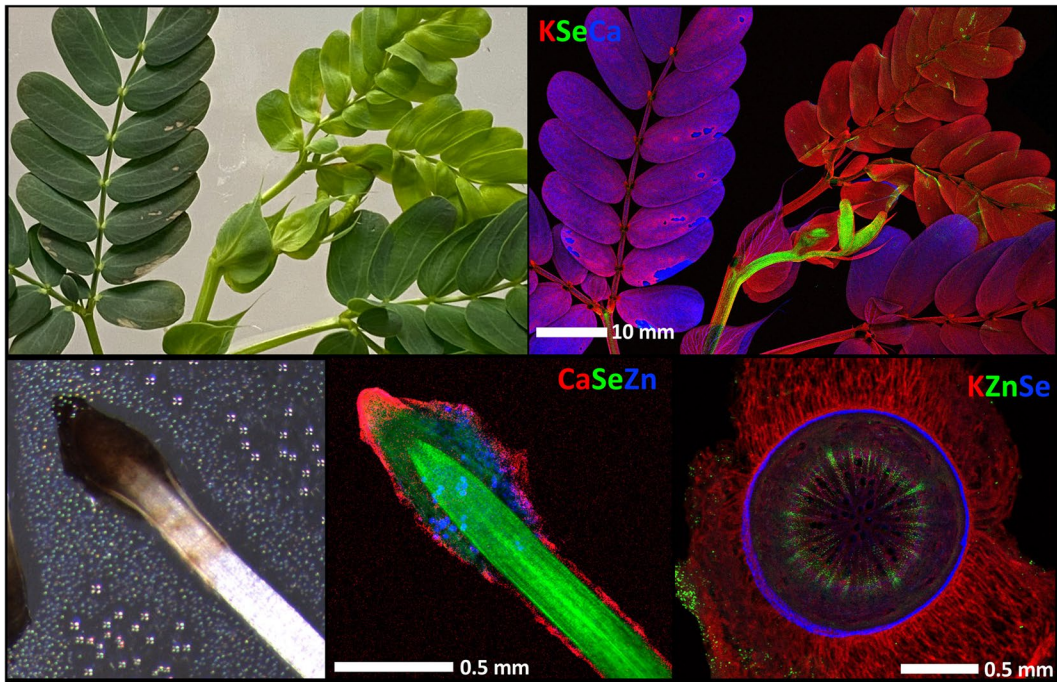


Fig. 10 Synchrotron μ XRF elemental maps showing the distribution of potassium, calcium, selenium and zinc in a hydrated leaves and roots of the selenium hyperaccumulator

Neptunia amplexicaulis grown in hydroponics and dosed with selenate-selenite salts. Unpublished data obtained at the XFM beamline of the Australian Synchrotron (ANSTO)

μ -XRF analysis were assessed, and in hydrated plant tissues dose-limits are 4.1 kGy, before damage occurs (Jones et al. 2020). The risk of radiation-induced damage can be avoided/minimised here by choosing appropriate measurement conditions, for example in a laboratory μ -XRF system the deposited radiation dose was just 6.6 Gy in an experiment on Mn hyperaccumulator species (Abubakari et al. 2021).

Considerations for transcriptomics analysis

Comparative transcriptomics analysis is a powerful tool to identifying transcripts involved in trace metal(loid) accumulation capacity or metal(loid) toxicity/tolerance in hyperaccumulator plants (Blande et al. 2017; de la Torre García et al. 2021). As such, it can be used to identify genes of interest among those differentially expressed between hyperaccumulator and non-accumulator species pairs, for example *Stanleya pinnata* versus *S. pinnata* (Wang et al. 2018) or contrasting *Noccaea caerulea* accessions (Halimaa et al. 2014). It is important in transcriptomics experiment that any toxicity or

deficiency are prevented in the controls, to avoid ‘unnatural’ non-metal specific high or low expression levels of the target genes. When studying metal-induced transcriptomic changes, it is desirable to maintain the same background nutrient solution in the controls and the metal/metalloid treatments, to prevent any unforeseen induction of transcriptomic changes other than through the treatment. Particularly in studies aimed at identifying tolerance-, or accumulation-correlated natural variation in transcript levels between species or con-specific populations, it may be preferable to use a dilute background solution that more or less mimics a ‘natural soil solution’, to avoid any masking of differences in gene expression involved in natural differential tolerance or accumulation capacities.

Typically, it is advantageous to use largely homozygous seedlings from inbred lines, or clones derived from cuttings (or tissue culture), instead of ‘bulk seed stock’ from natural populations. In general, culture in soil allows for transcriptomics analyses of shoots, but not of the roots, because it is virtually impossible to sufficiently decontaminate

soil-borne roots for transcriptomics analysis. Therefore, hydroponics is essential for root transcriptomics. Although in hyperaccumulator plants metal(loids)s are largely sequestered in the shoot, the root elongation test for metal tolerance still reflects the natural variation in Zn- and Cd-tolerance e.g., in *N. caerulescens* (Kozhevnikova et al. 2020), and there is convincing evidence that metal-imposed root growth inhibition in this species, as in the non-hyperaccumulator, *Silene vulgaris* (De Knecht et al. 1992; Harmens et al. 1993), is entirely attributable to toxic metal accumulation in the root itself, irrespective of the shoot response. Thus, to get a complete picture of metal tolerance in hyperaccumulator plants, analysis of the root transcriptome is indispensable.

In interspecific transcriptomics comparisons between hyperaccumulators and non-hyperaccumulators it is key to choose a (preferably genetically closely related) non-hyperaccumulator reference species. In studies with the model Zn/Cd hyperaccumulator *A. halleri*, non-hyperaccumulator congeneric *Arabidopsis* species are available, in particular *A. thaliana*, which has the additional advantage of being the best studied species among all seed plants. Another *Arabidopsis* (facultative) non-hyperaccumulator metallophyte (*A. arenosa*) may be chosen as a reference, as metallicolous Zn/Cd-hypertolerant populations are available for this species (Turisová et al. 2013; Preite et al. 2019). In physiological/biochemical and transcriptomics comparisons, the Zn/Cd/Ni hyperaccumulator model *N. caerulescens* (formerly *Thlaspi caerulescens*) is unfortunately without a suitable reference species. *Thlaspi arvense* has often been used as a non-hyperaccumulator reference for *N. caerulescens* (e.g., Richau and Schat 1999; Kozhevnikova et al. 2014), however, this taxon is not closely related to *N. caerulescens* (Koch and German 2013). Therefore, *Noccaea perfoliata* (formerly *Microthlaspi perfoliatum*) is increasingly used as a non-hyperaccumulator reference instead (Kozhevnikova et al. 2020). While *N. perfoliata* is non-metallicolous and lacks Zn/Cd hyperaccumulation or hypertolerance traits, all of its populations thus far tested, showed considerable Ni hyperaccumulation and Ni tolerance (Kozhevnikova et al. 2020), which may be an attribute of the whole *Noccaea* genus.

Conclusions

For any nutrient, both the minimally ‘sufficient’ concentration and the highest no-effect-concentration (NEC) for toxicity, can vary by orders of magnitude between different species, or even between conspecific populations. Therefore, it is not possible to define a ‘universal’ nutrient solution that is both sufficient and non-toxic for all plants, although it is often well possible, dependent on plant species under study and the research question to be addressed to ‘adapt’ commonly used ‘standard formulations’. However, to avoid any unforeseen toxicity phenomena, or to minimise the chance of potential nutrient concentration \times treatment interactions, investigators increasingly use dilute, ‘soil solution-like’ nutrient solutions. The use of hydroponics has proven to be a superbly powerful tool in studying the regulation of metal/metalloids in hyperaccumulator plants. In general, the choice for any particular background nutrient solution composition should not only be based on the deficiency and toxicity thresholds of the species or populations under investigation, but also on the scientific question to be addressed. Well-designed and executed hydroponics experiments can yield powerful insights in the homeostasis of essential and toxic trace elements, and this extends far beyond trace element hyperaccumulator plants. It is crucial to carefully evaluate the design parameters to suit the experimental purpose.

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Author contributions All authors contributed to writing of the manuscript.

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Data availability The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

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