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Copper, cadmium and nickel pollution inhibit growth and promote ascorbate catabolism in cell cultures of *Arabidopsis thaliana* and *Zea mays*

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Abstract

We established model systems for exploring the roles of symplastic and apoplastic ascorbate in heavy-metal-polluted dicot and monocot cells. Cell-suspension cultures of *Arabidopsis* and maize were treated with copper, cadmium or nickel; growth and ascorbate metabolism were measured. Growth was halved by ~80 μM Cu^{2+}, 90 μM Cd^{2+} or 1200 μM Ni^{2+} in *Arabidopsis*, and ~90 μM Cu^{2+}, 650 μM Cd^{2+} or 650 μM Ni^{2+} in maize. Cu^{2+} (128 μM) and Cd^{2+} (512 μM) caused partial loss of symplastic ascorbate, especially in *Arabidopsis*; Ni^{2+} (512 and 2048 μM) had moderate effects. Added apoplastic L-ascorbate (1 mM) was consumed by the cultures (half-life ~23 and 44 min in *Arabidopsis* and maize respectively), consumption rate being 3–6-fold increased by Cu^{2+}, Cd^{2+} and Ni^{2+} in *Arabidopsis*, and by Cu^{2+} in maize; Cd^{2+} and Ni^{2+} had relatively little effect on apoplastic ascorbate consumption in maize. Radioactivity from exogenous 1 mM L-[1-^{14}C]ascorbate remained extracellular; catabolites formed were dehydroascorbic acid, diketogulonate and oxalyl-threonates. In conclusion, suspension-cultured cells respond to heavy-metal stresses by maintaining symplastic ascorbate concentrations, which may beneficially scavenge symplastic reactive oxygen species (ROS). Apoplastic ascorbate is catabolised in metal-polluted cultures via several oxidative and non-oxidative reactions, the former potentially scavenging stress-related apoplastic ROS.

Key words: heavy metal stress, reactive oxygen species, ascorbate turnover, cell cultures, defence response, metal detoxification.

Subject classification codes: include these here if the journal requires them

Introduction

Plants can often acclimate in response to abiotic stresses such as heavy-metal pollution, heat, freezing, drought, flooding and salinity that would otherwise cause yield losses in crops and reproductive failure in natural environments (Boursiac et al. 2008; Mittova et al. 2015). Many abiotic stresses are accompanied by the adverse production

of reactive oxygen species (ROS), which can oxidise proteins (causing loss of enzyme activity), phospholipids (causing membrane leakiness) and DNA (causing mutation) (Wang et al. 2003). The ROS produced include hydrogen peroxide (H_2O_2), the hydroperoxyl radical ($HO_2^{\bullet}$) [and its ionised form the superoxide radical ($O_2^{\bullet-}$; $pK_a \approx 4.8$)], the hydroxyl radical ($^{\bullet}OH$) and singlet oxygen ($^1O_2$).

Cadmium is one of the most harmful pollutants (Kováčik et al. 2009) and is also a global threat to safe crop production (Grara et al. 2012; Farzadfar and Zarinkamar, 2012). Cd stress progressively damages maize (*Zea mays* L.) growth and chloroplast function (Xu et al. 2014). Cd toxicity caused a rapid rise in H_2O_2 and malondialdehyde, which further assist electrolyte leakage in leaves of maize. In response to Cd-induced ROS, increases occur in the activities of superoxide dismutase, peroxidase, catalase, ascorbate peroxidase and glutathione peroxidase (Anjum et al. 2015). Copper, although being an essential micronutrient, has a severe negative impact on plants when present in excess (Singh et al. 2007). The reduction in growth due to heavy metal stress is not a general response: for example, Bochicchio et al. (2015) found that treatment of *A. thaliana* roots with low doses of Zn^{2+} in hydroponics induced an increase in root length and branching. Nickel is another essential microelement that is toxic when in excess (Rathor et al. 2014); a high concentration of Ni^{2+} in the rhizosphere influenced *Arabidopsis thaliana* (L.) Heynh. leading to geotropism defects by suppressing root cell elongation without significantly affecting stem cell function (Lešková et al. 2020). Li et al. (2015) demonstrated a positive correlation between nucleolar damage and Ni^{2+} concentration. Furthermore, Sachan and Lal (2017) showed that excess Ni^{2+} causes osmotic imbalance, enzyme inhibition, cell structure disruption, wilting, reduced photosynthetic activity, oxidative stress and visible morbidity.

Plants possess several defence mechanisms against excess ROS, including antioxidants (ascorbate, glutathione and tocopherols) and ROS-consuming enzymes (e.g. superoxide dismutase, peroxidase and catalase) (Mendoza-Cózatl et al. 2005). For example, antioxidants provide a means of scavenging aluminium-induced ROS (Dutta et al. 2018) and Cd^{2+}-induced ROS (Mishra et al. 2019). Ascorbate is the major antioxidant able to mitigate environmental stresses; it also acts as a signalling molecule for cell division and cell expansion (Liso et al. 1984; Conklin and Barth 2004; Zhang et al. 2007; Venkatesh and Park 2012) and may contribute to cell-wall loosening (Green and Fry 2005; Airianah et al. 2016). It can increase tolerance of abiotic stresses, enhancing growth, photosynthesis, and resistance to oxidative damage, and help to maintain photosynthetic pigments. The role of ascorbate [and potentially also un-ionised ascorbic acid ($pK_a \approx 4.2$) in acidic cellular compartments such as the apoplast — the extra-protoplasmic aqueous solution which permeates the cell wall] as an antioxidant capable of scavenging heavy-metal-generated ROS is well established; in particular, it is important to consider that ascorbate plays different roles in different sub-cellular compartments: it is biosynthesised in the mitochondria, functions as an antioxidant in the cytosol and (especially) in the chloroplasts, it serves as a precursor of oxalate and tartrate in certain tissues, and it also plays important roles, including as a pro-oxidant, in the apoplast (Fry 1998; Smirnoff 2000; Sanmartin et al. 2003; Foyer and Noctor 2011; Qian et al. 2014).

In living plants, ascorbate is constantly synthesised and broken down, and its steady-state pool size is the balance of these opposing processes. Most ascorbate is located in the cytoplasm, but in the order of 10% may be secreted into the apoplast (solution which permeates the cell wall) (Pignocchi and Foyer 2003; Zechmann 2011; Sharova et al. 2020), where it constitutes a primary line of defence against external

oxidants (Fig. 1). Generally, the redox buffering capacity of the cell apoplast is dependent on the presence of the ascorbate pool (Pignocchi et al. 2006). Apoplastic ascorbate breakdown is initiated by its oxidation — initially to semidehydroascorbate, which immediately disproportionates to ascorbate plus a relatively stable oxidation product, dehydroascorbic acid (DHA). As shown in Fig. 1, DHA is a branch-point in ascorbate catabolism (Green and Fry 2005): it can be further oxidised to oxalate and its threonate esters (Parsons et al., 2011) and/or hydrolysed to diketogulonate (DKG). DKG can then generate 2-carboxy-L-*threo*-pentonolactones (CPLs), 2-carboxy-L-*threo*-pentonate (CPA), 2-oxo-L-*threo*-pentonate (OTP; '2-keto-L-xylonate') and threonate (Dewhirst and Fry 2018; Dewhirst et al. 2020. The various oxidations mentioned above (red arrows in Fig. 1) are of great interest because they involve reaction of the organic substrate with ROS, which are thereby 'scavenged', potentially conferring resistance against the adverse effects of environmental stresses.

In the present work, we explore the levels and turnover of ascorbate in response to sub-lethal but growth-inhibiting concentrations of Cu^{2+}, Cd^{2+} and Ni^{2+}. Cell-suspension cultures were employed because they can easily be bathed in a uniform and precisely known concentration of pollutants, and radiolabelled ascorbate can be instantaneously applied (for monitoring turnover) to all cells in the population — unlike the situation in intact plants, which possess numerous distinct cell types and are shielded by a cuticularised epidermis.

Cell cultures of two plant species were tested in these experiments: maize, a monocotyledonous C_4 crop of tropical and temperate climates widely used in studies of stress physiology; and the best-studied model dicotyledonous plant, *Arabidopsis*. Maize is very sensitive to pollution by several metals, suggesting its potential use as a test plant to evaluate contaminated soils (Romdhane et al. 2021). A comprehensive

knowledge of plant–metal interactions is essential for minimizing the impact of risks accompanying the addition of metals into the food chain (Benavides et al. 2005; (Bielen et al. 2013). The results enhance our understanding of the functions of ascorbate in heavy-metal tolerance in both plant species.

Material and Methods

Preparation of the formate salts of copper, cadmium and nickel

Stock solutions of formate salts of Cu^{2+}, Cd^{2+} and Ni^{2+} were as prepared from commercial $CuCl_2$, CdO and $NiSO_4$ respectively. CdO (1 mmol) was dissolved in 5 ml water containing 5 mmol formic acid, and the solution was filtered, dried in a SpeedVac (Thermo Electron Co-operation) and re-dried from water four more times, removing the excess formic acid; the cadmium formate was re-dissolved in water, giving a 20 mM stock solution. A solution of $CuCl_2$ (1 mmol in 25 ml water) was mixed with 2.2 mmol NaOH, and after 4 h the precipitated $Cu(OH)_2$ was thoroughly water-washed, bringing the solution to neutrality; the resulting 1 mmol of $Cu(OH)_2$ was treated with 5 mmol formic acid and processed as for cadmium, yielding a 20 mM copper formate stock solution. Nickel formate was likewise prepared from 1 mmol $NiSO_4$ via insoluble $Ni(OH)_2$. The three formate salts were checked for concentration by high-voltage paper electrophoresis (HVPE) followed by staining with bromophenol blue (Fry 2017).

Cell suspension cultures

Maize (*Zea mays* L., Black Mexican sweetcorn) cell-suspension culture was donated by Dr I. Moore (Department of Botany, University of Edinburgh) and maintained on Murashige & Skoog, (1962) salts containing 20 g l^{-1} sucrose, 0.1 mg l^{-1} aneurine-HCl, 0.5 mg l^{-1} pyridoxine-HCl, 0.5 mg l^{-1} nicotinic acid, 300 mg l^{-1} L-asparagine and 5.0

mg l^{-1} 2,4-dichlorophenoxyacetic acid, final pH 6.0 (200 ml culture per 500-ml flask), incubated at 25°C under continuous low-intensity fluorescent lighting (~25 µmol m^{-2} s^{-1}) with orbital shaking (150 rpm), and sub-cultured weekly by 10-fold dilution.

Arabidopsis thaliana (L.) Heynh. (hereafter '*Arabidopsis*') cell-suspension cultures were grown in the medium of May and Leaver (1993) with glucose in place of sucrose, and sub-cultured weekly at the same volume, shaking speed, light and temperature as maize.

Treatment of mini-cultures with heavy metals

For testing metal ion effects, 100 ml of 7-day-old *Arabidopsis* or maize culture was diluted to 300 ml with fresh medium, and then 5-ml aliquots of these '0-day-old' cultures were aseptically transferred into loosely capped sterile 60-ml cylindrical polycarbonate beakers (Bibby Sterilin Ltd, Stone, Staffordshire, UK), referred to as 'mini-cultures' (O'Looney and Fry 2005a). An appropriate volume (0–512 µl) of 20 mM Cd^{2+} formate or Ni^{2+} formate or 10 mM Cu^{2+} formate was added to give the final concentrations required. The mini-cultures were incubated with shaking at 100 rpm under aseptic conditions for up to 1 week.

Growth measurements

Settled cell volume (SCV) was determined after allowing the cells from a 5-ml mini-culture to sediment under 1× g in a graduated 15-ml plastic tube. Packed cell volume (PCV) was then measured on the same cells after centrifugation at 1500 × *g* for 5 min (Fry and Street 1980). The cells' susceptibility to compaction by centrifugation (SCC) was calculated as [(SCV − PCV) / SCV] (Fry and Street 1980). For fresh weight measurements, the mini-culture was filtered through Miracloth, gently squeezed with

uniform pressure, then weighed.

Measurement of endogenous and exogenous ascorbate concentrations

Endogenous ascorbate: a 5-ml mini-culture was filtered on Miracloth at a specific age after inoculation. A weighed portion of cells (~120 mg fresh weight) was added to 480 µl 2% (w/v) metaphosphoric acid, and shaken at 20°C for 30 min, then centrifuged for 2 minutes at 14500 rpm; next, 200 µl of the supernatant was titrated for ascorbate by the progressive addition of 5-µl shots of 3.73 mM DCPIP (2,6-dichlorophenolindophenol) until the dye was no longer decolourised and a visible pink colour persisted for at least 10 s. Authentic ascorbic acid in 2% w/v metaphosphoric acid gave a linear standard curve (number of 5-µl shots required to impart a pink colour to 0–16 mM ascorbate).

Exogenous ascorbate: 5-ml mini-cultures (age ‘0 d’; i.e. 1 vol. of 7-d culture mixed with 2 vol. fresh medium, immediately dispensed at 5 ml per polycarbonate beaker), supplemented or not with heavy metals, were then immediately supplemented with exogenous ascorbate (final concentration 1 mM) (O’Looney and Fry 2005). The ascorbate was added from a 100 mM stock solution of ascorbic acid pre-adjusted with NaOH to the pH of the respective cultures: 5.5 for maize and 5.6 for *Arabidopsis*. After a further ‘0’–240 min incubation under standard conditions (in loosely capped Sterilin beakers with gentle shaking), 200-µl samples of cell-free medium were removed (after sedimentation of the cells for ~2 min) and DCPIP-titrated for remaining extracellular ascorbate as above. The ‘0’-min sample was taken ~2 min after addition of the ascorbate.

Fate of radioactive L-ascorbate

The fate of exogenous L-[1-^{14}C]ascorbate was also examined quantitatively and

qualitatively.

For a quantitative study, 5-ml ‘0-day’ mini-cultures were set up in polycarbonate beakers and, after 5 min, treated with or without heavy metals. After a further 5 min, [^{14}C]ascorbate (0.37 kBq, pre-mixed with 50 µl 100 mM non-radioactive ascorbate adjusted to pH 5.5 with NaOH) was then added to each mini-culture, giving a final concentration of 1 mM at 74 Bq/ml, and incubation was continued under standard conditions. At intervals (0–6 h), 320 µl of cell-free medium was taken: 200 µl was DCPIP-titrated for remaining ascorbate, and 100 µl was assayed for ^{14}C (theoretical maximum in the absence of ^{14}C uptake by the cells = 7.4 Bq, i.e. ~400) by scintillation-counting.

For a qualitative study of the fate of exogenous [^{14}C]ascorbate (based on the method of Green and Fry 2005), we produced 20-µl 0-day ‘micro-cultures’ (i.e. immediately after mixing 7-d cultures with 10 volumes of fresh medium) in loosely capped, broad-bottomed 2-ml Eppendorf tubes, and after 5 min we added 1.3–8.1 µl of either water or a heavy-metal solution to give the desired final concentration. After a further 5 min with gentle shaking, we added 5 µl of 5 mM ascorbate (Na^+, pH 5.5) containing 500 Bq [1-^{14}C]ascorbate, to give a final concentration of 1 mM ascorbate. After ‘0’, or 240 min, the cell-free medium (~25 µl) was mixed with 5 µl 90% formic acid and stored frozen for later electrophoresis.

High-voltage paper electrophoresis (HVPE)

For HVPE, 15 µl of the formic acid-stopped sample (theoretical maximum ~250 Bq) was dried on Whatman No. 3 paper and electrophoresis was conducted in pH 2.0 buffer (formic acid/acetic acid/water, 2:7:71 by vol.) at 2.5 kV for 50 min. Methodological details were as described (Fry 2020). Each radioactive sample was premixed with a

trace of Orange G as internal marker. An external marker mixture contained 20 μg each of L-ascorbic acid, L-tartaric acid and L-threonic acid, which were stained with $AgNO_3$ (Fry 2000). The unstained, radioactive part of the paper was autoradiographed on Kodak BioMax MR-1.

Reproducibility

In most of the reported experiments (Figs. 2, 3, 4), datapoints are accompanied by error bars representing the range (N=2), with symbols plotted at the means, interconnected by straight lines; we have not attempted to mathematically fit smooth curves, but nevertheless clear trends are visible. Therefore, it is not appropriate to offer values for statistical significance. In some cases (Figs. 5 and S3), we did mathematically fit smooth curves, on which basis standard errors for the measured half-lives are reported. Replicate experiments gave comparable data. For example, inter-experimental reproducibility can be judged by comparison of Fig. 5 with a repeat experiment (Fig. S3), via the ± values quoted in Table S1.

Results

Selection of suitable concentrations of Cu^{2+}, Cd^{2+} and Ni^{2+}

Mini-cultures (5 ml) of *Arabidopsis* and maize grew 5–6-fold (PCV basis) during 1 week's incubation in the absence of pollutants (Fig. 2a,b). Exposure to heavy metal ions inhibited this growth: in *Arabidopsis*, the 1-week increase (ΔPCV) was halved by Cu^{2+}, Cd^{2+} and Ni^{2+} at approximately 80, 90 and 1200 μM respectively (IC_{50}, i.e. concentration giving 50% inhibition); in maize the corresponding IC_{50} values were ~90, 650 and 650 μM. Thus, in both plant species, Cu^{2+} was about an order of magnitude more toxic than Ni^{2+}; however, maize was much more Cd^{2+}-tolerant than *Arabidopsis*. At concentrations only moderately above the IC_{50}, growth was stopped completely: in

Arabidopsis, the 1-week increase in PCV was reduced to zero (i.e., ΔPCV = 0) by Cu^{2+}, Cd^{2+} and Ni^{2+} at about 100, 250 and 1700 µM respectively; in maize the required concentrations were 120, 1200 and 2000 µM respectively. At metal ion concentrations exceeding twice the IC_{50} values, the cells not only failed to increase in PCV but also often shrank to cell volumes below the time-zero inoculum (i.e., ΔPCV < 0%; Fig. 2a,b), suggesting cell damage.

The heavy metal ions also tended to increase the cells' susceptibility to compaction by centrifugation (SCC; Fig. 2c,d), suggesting that the architecture of the cell aggregates became looser; this effect was even noticeable in *Arabidopsis* treated with moderate Cd^{2+} concentrations (~30–60 µM) that had relatively little effect on growth.

In the light of the PCV data, we chose for more detailed investigations 128 and 512 µM for Cu^{2+}, and 512 and 2048 µM for both Cd^{2+} and Ni^{2+}, thus covering a 4-fold range close to and above the concentrations necessary to inhibit growth.

Symptoms of metal toxicity were also observed visually. In *Arabidopsis* mini-cultures, a gradual change in colour was observed in metal-treated mini-cultures, from green (healthy) to pale yellow (morbid) (Fig. S1). Cu^{2+} obliterated chlorophyll more rapidly (data not shown) and more completely (Table 1) than Cd^{2+} and Ni^{2+} of the same concentrations. In maize, the colour change was from cream (healthy) to dark brown; again, Cu^{2+} toxicity was more evident than Cd^{2+} and Ni^{2+} (Fig. S2; Table 1).

Time-course of growth of metal-polluted mini-cultures

In the absence of heavy metals, 5-ml mini-cultures of both *Arabidopsis* and maize increased in fresh weight almost linearly for 1 week, reaching roughly 3× the zero-time value (Fig. 3a,b). In both species, the low Ni^{2+} dose (512 µM) had little effect on growth in fresh weight, but the high dose (2048 µM) blocked growth, with the cells neither gaining nor losing fresh weight. Low Cd^{2+} (512 µM) initially permitted some growth

but later caused the fresh weight to diminish (*Arabidopsis*) or plateau (maize); high Cd^{2+} (2048 μM) also briefly permitted growth but later caused a weight loss. Low Cu^{2+} (128 μM) initially permitted slight growth in *Arabidopsis*, but then caused weight loss; high Cu^{2+} (512 μM) caused weight loss throughout the week. Growth as measured by PCV followed similar trends (Fig. 3c,d).

Endogenous ascorbate in metal-polluted mini-cultures

In view of the proposed role of ascorbate as an anti-oxidant providing protection against heavy-metal-induced ROS, we measured the endogenous symplastic ascorbate in Cu^{2+}-, Cd^{2+}- and Ni^{2+}-treated mini-cultures during the course of 1 week's growth (Fig. 4). Unpolluted mini-cultures contained 0.5–1.5 mM symplastic ascorbate (average concentration in whole extracted cell-sap). In both *Arabidopsis* and maize, the concentration peaked at about 1.5 mM in mid-growth, then diminished.

The lower metal ion concentrations (128 μM Cu^{2+} and 512 μM Cd^{2+} and Ni^{2+}) affected ascorbate levels differently in *Arabidopsis* versus maize (Fig. 4). In maize, the lower concentrations of all three metals had relatively little effect on ascorbate concentration (Fig. 4b), indicating that maize cells maintained ascorbate synthesis (and reversed dehydroascorbic acid production) despite the metals' adverse effects on growth. In contrast, *Arabidopsis* mini-cultures suffered substantial ascorbate loss at the low Cu^{2+} and Cd^{2+} (but not Ni^{2+}) concentrations (Fig. 4a), indicating that *Arabidopsis* was less adept than maize at maintaining anti-oxidant levels as a defence against the stress of heavy metal pollution.

Relative to non-polluted controls, both concentrations of Ni^{2+} increased the endogenous ascorbate in maize by the end of the culture period, suggesting that ascorbate biosynthesis was activated as a defence response to the stress of Ni^{2+} pollution. In

Arabidopsis, low Ni^{2+} also substantially raised ascorbate, though high (toxic) Ni^{2+} concentrations gradually diminished it.

Likewise, low Cd^{2+} permitted the maize cells to retain a high ascorbate content, but high Cd^{2+} caused ascorbate loss in both plant species.

The pattern seen with Cd^{2+} also applied to Cu^{2+}. However, in the latter case in particular, it is important to consider the possibility that some of the added Cu^{2+} (traces of which may have remained on or in the cells despite washing on Miracloth) caused autoxidation (Buettner and Czapski 1986) of ascorbate during extraction and titration. Nevertheless, our data suggest that this is unlikely because ascorbate was readily detectable at the first two time-points, '0' and 3 h after addition of Cu^{2+}, in both plant species. The severe loss of ascorbate in high-Cu^{2+} media occurred only after the Cu^{2+} had caused a substantial decrease fresh weight, probably indicating cell death. Thus, the decrease in ascorbate seen in high-Cu^{2+} media was not principally an artefact due to autoxidation of extracted ascorbate.

Consumption of apoplastic ascorbate in metal-polluted cultures

A small proportion of the ascorbate in plant tissues is located in the apoplast, where it may serve a beneficial anti-oxidant role (Fig. 1), scavenging ROS (Sanmartin et al. 2003), while being itself oxidised. To investigate this, we added ascorbate to 1 mM with or without heavy-metal pollution, and monitored the kinetics of its 'consumption' for up to 4 h (Fig. 5 and Fig. S3 — in the absence and presence, respectively, of a trace of radiolabelled ascorbate). Table S1 collates data on the longevity of added extracellular ascorbate in both species, in the presence of zero, low and high concentrations of the three heavy metal ions, and in the two independent experiments presented in Fig. 5 and Fig. S3.

In unpolluted mini-cultures, the extracellular ascorbate gradually disappeared; the mean lifetime of ascorbate [τ, the time when 36.8% (= 100/e) remained] was roughly 30 and 60 min in *Arabidopsis* and maize respectively. The spent media contained no detectable ascorbate if none had been added, showing that any ascorbate secreted by the cells was quickly oxidised or re-absorbed.

In *Arabidopsis*, all three tested metals promoted the consumption of ascorbate (Fig. 5a; Fig. S3a), suggesting that the ascorbate was being utilised to scavenge ROS.

In maize, high Cd^{2+} sometimes delayed the consumption of ascorbate (Fig. 5b). In other experiments, this delaying effect of high Cd^{2+} was not observed (Fig. S3). Ni^{2+} (both concentrations) also had little effect on ascorbate consumption by maize. However, Cu^{2+} (both concentrations) strongly promoted the consumption of ascorbate by maize mini-cultures.

Thus, while there were appreciable differences between the behaviour of *Arabidopsis* and maize, the most consistent trend was a promotion of ascorbate consumption (potentially acting as an anti-oxidant) in the presence of heavy metal ions, especially Cu^{2+}.

The heavy metals could have been acting as a stress, inducing the cells to produce ROS, which would oxidise extracellular ascorbate; alternatively, the metals themselves may have catalysed the 'autoxidation' (by dissolved O_2) of extracellular ascorbate. To distinguish these possibilities, we incubated cell-free spent medium with heavy metals plus ascorbate (Fig. S4). Cu^{2+} drastically promoted ascorbate oxidation, confirming its ability to catalyse autoxidation, and this oxidation was even more rapid than in the presence of cells (Fig. 5 and Fig. S3). We suggest that the cells, when present, absorbed the Cu^{2+}, diminishing its ability to promote the autoxidation of extracellular ascorbate. On the other hand, Cd^{2+} and Ni^{2+} did not promote ascorbate autoxidation; in contrast,

they inhibited it compared with unpolluted controls (Fig. S4). This was presumably due to the ability of Ni^{2+} and especially Cd^{2+} to inhibit the ascorbate oxidase present in the spent medium.

The carbon of apoplastic [^{14}C]ascorbate remains extracellular

The disappearance of exogenous apoplastic ascorbate in cell cultures, as assayed by DCPIP titration of spent media, could be due to extracellular catabolism (Fig. 1) and/or uptake of ascorbate and its catabolites into the cells. The above experiments did not discriminate between these two possibilities. We therefore conducted a similar experiment with 0-d-old mini-cultures, supplemented by ~1 mM ascorbate plus a trace of radiolabelled exogenous ascorbate (too little to perceptibly affect the total exogenous ascorbate concentration). Catabolism alone would not result in a loss of extracellular ^{14}C (unless $^{14}CO_2$ was produced), whereas uptake into the cells would.

DCPIP titration confirmed the previously noted trends of total extracellular ascorbate loss except that, in the present experiment, high Cd^{2+} did not preserve the ascorbate in maize mini-cultures (Fig. S3). However, whereas the ascorbate disappeared from the medium (Fig. S3), the ^{14}C did not (Fig. 6). Therefore, the heavy-metal-promoted 'consumption' of apoplastic ascorbate was due to extracellular catabolism, and the organic degradation products were not appreciably taken up by the cells during the period of observation (4–6 h). Furthermore, the catabolites did not include volatile products such as $^{14}CO_2$, which would not have been detected by scintillation-counting of spent medium samples. Thus, the DKG → OTP → threonate pathway (Fig. 1) was undetectable since the DKG → OTP step would have released the ^{14}C from [1-^{14}C]DKG, derived from the fed [1-^{14}C]ascorbate.

Qualitative analysis of apoplastic ascorbate catabolites

To investigate what degradation products were formed from extracellular ascorbate by metal-polluted cells, we supplied 25-µl micro-cultures with extracellular 1 mM [1-^{14}C]ascorbate and analysed the extracellular products by HVPE in a buffer at pH 2.0 (Fig. 7).

In pH 2.0 buffer, many organic acids (e.g. ascorbate and threonate) carry a negligible charge and are therefore almost immobile electrophoretically. Only those organic acids with an unusually low pK_a can migrate towards the anode; however, this includes many important ascorbate catabolites such as DKG, oxalate, OxT, cOxT, OTP, CPA and CPL (Dewhirst and Fry 2018; Dewhirst et al. 2020). In both *Arabidopsis* and maize micro-cultures, the majority of the ^{14}C was present in electrophoretically immobile products (ascorbate and/or dehydroascorbic acid, not resolved from each other, but clearly not ascorbate by 240 min; Fig. S3). A minority of the ^{14}C was present in oxalyl threonates (mainly 4-OxT in *Arabidopsis*; a mixture of 3-OxT and 4-OxT in maize; plus a trace of cOxT), their concentrations not changing appreciably with time or in the presence of heavy metals. In unpolluted micro-cultures of both species, DKG, the hydrolysis product of DHA, gradually appeared (more after 240 min than at time zero); the metal ions, especially Cu^{2+} but also Cd^{2+} and Ni^{2+}, tended to promote DKG production (Fig. 7).

Discussion

Cu^{2+}, Cd^{2+} and Ni^{2+} each inhibited the growth of *Arabidopsis* and maize cell-cultures, and there was evidence for effects on cell-aggregate morphology (as judged by SCC). According to PCV measurements, Cu^{2+} and Cd^{2+} were ~15× more inhibitory than Ni^{2+} in *Arabidopsis*, whereas in maize Cu^{2+} was ~8× more inhibitory than both Cd^{2+} and Ni^{2+}. In general, the effects of heavy metal stress were greater in *Arabidopsis* than in maize cell-cultures. Others have also reported that metal uptake and effects vary by

plant species and by organ (Chen et al. 2010; Wu et al. 2015; Jung et al. 2016; Jung et al. 2018). Although we cannot explain the physiological/biochemical basis for the maize/ *Arabidopsis* differences, there is clearly no 'uniform' response to heavy-metal pollution applicable across the plant kingdom.

Our data also show that heavy metals caused chlorosis in *Arabidopsis* cultures and an accumulation of dark brown pigments in maize cultures (Table 1). Similar detrimental effects were observed in wheat leaves exposed to 100 µM Ni^{2+}, which caused chlorosis and necrosis within 3 d (Gajewska and Skłodowska 2007). Cd^{2+} also caused chlorosis in maize plants (Lagriffoul et al. 1998). Heavy metal-induced chlorosis adversely affects photosynthesis (Sharma et al. 2020) and 100 µM $CdCl_2$ caused programmed cell death in *Arabidopsis* cell-cultures (De Michele et al. 2009). Other physiological maladies might be a secondary effect caused by mineral deficiency (Hédiji et al. 2015). For example, Cd^{2+} competes with various essential elements such as Zn^{2+}, Cu^{2+} and Ca^{2+}, adversely affecting enzyme activities, DNA repair, cellular redox states and signal transduction (Valko et al. 2016).

We estimated the metal concentrations that led to almost 100% growth inhibition, and then examined various effects of these and 4-fold higher concentrations. Similar findings were reported by (Abdel Latef et al. 2020), where increasing Cu^{2+} (100 to 500 µM) concentration reduced growth traits and photosynthetic pigments in maize plants. In *Arabidopsis*, Cd^{2+} significantly reduced chlorophyll and growth (Szopiński et al. 2019). However, lower concentration of Cd^{2+} did not significantly affect the growth of maize (Ling et al. 2017); similar findings are described in the current study (Table 1; Fig. 2).

Our experiments employed cell-suspension cultures rather than whole plants. Clearly, there may be physiological differences between plant cells in culture and those *in*

planta, and therefore our observations cannot be directly equated with the responses of whole plants to heavy-metal pollution. Nevertheless, cell cultures have several key advantages: for example, all the cells in a culture are essentially identical morphologically and biochemically, rather than being a collection of different cell-types (from meristems, mesophyll, cortex, pith, epidermis, phloem etc.) and thus a single consistent response to metals can be expected rather than a range of different responses; secondly, it is certain that the metal ion concentration applied to a culture is the concentration initially experienced by all the cells present in the population, whereas in intact plants the concentrations experienced will be lower than the concentrations applied because of the barrier presented by the cuticle (in shoots) or Casparian strip (in roots); and thirdly, in cell cultures it is particularly simple to collect the apoplastic fluid (= culture filtrate) for apoplastic ascorbate analysis whereas in planta it is necessary to invasively collect samples, e.g. by vacuum infiltration and centrifugation (Lohaus et al. 2001; Sanmartin et al. 2003).

In maize cultures, the lower (but growth-inhibiting) Cu^{2+}, Cd^{2+} and Ni^{2+} concentrations (125, 512 and 512 µM respectively) had relatively little effect on endogenous symplast ascorbate, suggesting that the cells acclimated to these metals by maintaining ascorbate production (Fig. 4b). Symplastic ascorbate may be beneficial in scavenging ROS, which are often generated in response to stresses including heavy metal pollution (Bielen et al. 2013). Ascorbate acts as a substrate for regenerating essential antioxidants, thus maintaining the cellular redox state (Jozefczak et al. 2012). Moreover, the ascorbate pool usually varies in different organs of the same plants exposed to Cu^{2+} stress (Thounaojam et al. 2014). Metals have often been shown to regulate the ascorbate pool, and Cd^{2+}/ascorbate interaction is well established (Wu et al. 2015; Aravind and Prasad 2005; Romero-Puertas et al. 2007; Chao et al. 2010; Shen et al. 2012). Higher

concentrations of Cu^{2+} and Cd^{2+} (512 and 2048 μM respectively) decreased endogenous ascorbate, albeit not to zero, and Ni^{2+} even caused maize cells to produce more ascorbate than unpolluted controls (Fig. 4b). In addition, ascorbate may chelate toxic metals (Jozefczak et al. 2012). Our observations suggest that the cells prioritised ascorbate production under ROS-generating stressful conditions.

Ascorbate levels were generally more susceptible to heavy-metal ions in *Arabidopsis* than in maize cultures, corresponding with the greater susceptibility of *Arabidopsis* to growth inhibition by the metals. Cu^{2+} and Cd^{2+} at both tested concentrations diminished ascorbate pools in *Arabidopsis* (Fig. 4), Cu^{2+} being more effective despite being tested at 4× lower concentrations than Cd^{2+}. Nevertheless, the data still gave the impression that whilever the cells remained alive they prioritised ascorbate production (or retention) under these growth-inhibiting, metal-polluted conditions. Ni^{2+} did not strongly decrease ascorbate concentration and may even have increased it when added at 512 μM (Fig. 4a), in agreement with the findings of Gajewska and Skłodowska (2007) and Baccouch et al. (1998).

A small proportion of a plant's ascorbate is located in the apoplast, which would equate to the spent medium in cell-suspension cultures, where it may be beneficial in scavenging free radicals (Zechmann 2011; Venkatesh and Park 2012; Sharova et al. 2020). The apoplastic ascorbate:DHA ratio was diminished in Cd^{2+}-treated barley roots (Bočová et al. 2012). Indeed, if apoplastic ascorbate serves as a ROS-scavenging antioxidant, it will itself be oxidised — initially to monodehydroascorbate, then to the first stable product (DHA), and later to a range of downstream products, some of them also via ROS-scavenging reactions (Fig. 1) (Parsons et al. 2011; Dewhirst et al. 2020; Dewhirst and Fry 2018). To investigate the fate of apoplastic ascorbate, we monitored the cultures' ability to consume exogenous ascorbate. 'Consumption' of non-

radiolabelled ascorbate could include extracellular oxidation and/or uptake into the symplast. Metal pollution indeed promoted ascorbate consumption (Fig. 5, Fig. S3), as predicted if the ascorbate was being sacrificially oxidised as a means of scavenging metal-induced ROS. However, it was clear that the heavy metal ions were capable of exerting two antagonistic effects: (i) these pollutants stressed the cells, thus inducing the production of ROS which promoted ascorbate consumption; and (ii) they inhibited the activity of extracellular ascorbate oxidase, thus delaying ascorbate oxidation. The net outcome was the balance between these two conflicting effects. This may explain why, in the presence of cells, high Cd^{2+} promoted ascorbate consumption in some experiments [Fig. S3b; with (i) predominating], and inhibited it in others [Fig. 5b; (ii) predominating].

Ascorbate 'consumption' could represent its oxidation to dehydroascorbic acid (and downstream products) and/or uptake by the cells. We showed by radiolabelling that the exogenous ascorbate and any degradation products were not being perceptibly taken up by the cells or oxidatively decarboxylated (releasing $^{14}CO_2$ from carbon-1); on the contrary, essentially all the ^{14}C of the supplied [1-^{14}C]ascorbate remained in non-volatile form in the culture media (Fig. 6). Thus, the 'consumption' noted above was due to extracellular catabolism (with the products remaining in the apoplast). In contrast, Akram et al. (Akram et al. 2017) showed that ascorbate is oxidised to DHA, which is then transferred to cytoplasm for glutathione-mediated reduction and finally re-export of ascorbate into cell apoplast. Therefore, the data in Fig. 5 and Fig. S3 represent ascorbate oxidation, and in the process the ascorbate must be reducing something else, such as ROS (Sharova et al. 2020). Although we were not able to detect any naturally occurring extracellular ascorbate (unsurprisingly in view of its short half-life, in the order of 1 h; Fig. 5, Fig. S3), we conclude that the metal-polluted cells were able to use

any available apoplastic ascorbate to help protect against pollutant-generated ROS. Apoplastic ascorbate has been characterised as a major redox buffer (Pignocchi et al. 2006), which participates in numerous physiological phenomena, for example cell division, cell elongation, and cell defence.

In vitro, ascorbate yields different catabolites in the presence of different ROS (Dewhirst and Fry 2018) (Fig. 1). Therefore, as a contribution to investigating the nature of the ROS occurring under conditions of metal pollution, we used HVPE to identify the products generated *in vivo* from extracellular [1-^{14}C]ascorbate. After 6 hours' incubation, most of the ^{14}C was present in the form of [^{14}C]DHA (Fig. 7), indicating the presence of unspecified ROS. Traces of [^{14}C]OxT isomers, products of the further oxidation of DHA, were also detected, but scarcely more than were present in the time-zero non-polluted samples ('C 0h' in fig. 7), suggesting that H_2O_2 was not a major ROS in our cell-culture system (Fig. 1). Apart from the significant role of the ascorbate redox status in the apoplast, the ascorbate:DHA ratio has been considered correspondingly important to regulate guard cell signalling and stomatal movement (Chen et al. 2010). In view of previous studies, the differential effects of metals on ascorbate and DHA reported in current study may be extrapolated to whole-plant level. Several of the metal treatments produced elevated levels of DKG, the hydrolysis product of DHA. It is not clear why heavy metals promoted hydrolysis, but (non-oxidative) DKG production *per se* would not help to scavenge ROS. Downstream products of DKG (CPL isomers and CPA), also generated by non-redox reactions, were undetectable. Conversion of DKG to OTP is an oxidising reaction (observed *in vitro* in the presence of H_2O_2 or $^1O_2$) which could potentially scavenge ROS; however, the conversion of [1-^{14}C]DKG to OTP would release the radioactivity in the form of volatile $^{14}CO_2$, whereas negligible ^{14}C loss was observed. In summary, extracellular

ascorbate was sacrificially oxidised *in vivo* by the ROS produced in the presence of heavy-metal pollutants, and by far the major oxidative reaction occurring in this context was ascorbate → DHA. The failure of the DHA to be oxidised to oxalates and the failure of DKG to be oxidised to OTP suggests that H_2O_2 was not an abundant ROS in this system (Dewhirst and Fry 2018). On the contrary, our observations support $O_2^{\bullet-}$ as the main ROS present in heavy-metal-polluted *Arabidopsis* and maize cultures.

Conclusions

In the presence of heavy-metal pollutants, plants often respond by forming ROS, leading to oxidative stress and affecting growth, chlorophyll retention and ascorbate levels. In the presence of the metal ions tested here at growth-inhibitory concentrations, the cells, whilever they remained alive, appeared to prioritise the maintenance (biosynthesis and/or regeneration) of ascorbate — proposed to serve as an anti-oxidant that scavenges heavy-metal-generated ROS. Apoplastic ascorbate was metabolically labile (half-life ~1 h) in healthy cell cultures; it was more rapidly oxidised in the presence of many of the metal-stress conditions tested, indicating that it served to scavenge ROS. The nature of the extracellular ascorbate oxidation products detected suggested that the major apoplastic ROS generated in the presence of Cu^{2+}, Cd^{2+} and Ni^{2+} was superoxide rather than H_2O_2. This work adds to our understanding of heavy-metal toxicity and the roles of ascorbate, and, by highlighting differences between maize and *Arabidopsis*, it emphasises that more research is required on different plant taxa in the quest for generalisations.

Authors' contribution

SCF designed the research. FF performed most of the experiments. FF and SCF wrote the manuscript.

Declaration of Competing Interest

There are no competing interests to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at....................

Figure S1: Settled cell volume (SCV) and packed cell volume (PCV) of *Arabidopsis* cultures after 7 days' incubation in the presence of heavy metal ions.

Figure S2: Settled cell volume (SCV) and packed cell volume (PCV) of maize cultures after 7 days' incubation in the presence of heavy metal ions.

Figure S3: Short-term time-course of effect of heavy metal ions on consumption of exogenous ascorbate in the presence of a trace of [^{14}C]ascorbate.

Figure S4: Effect of heavy metal ions on the oxidation of ascorbate in cell-free spent medium.

Table S1: Time taken for cultures cells to consume extracellular 1 mM ascorbate in the presence of heavy metals.

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Table 1

Colour changes observed in mini-cultures of *Arabidopsis thaliana* (*Arabidopsis*) and *Zea mays* (maize) after 7 d incubation with or without heavy metals.*

Metal ion concentration (µM)	*Arabidopsis*			Maize		
	Cu^{2+}	Cd^{2+}	Ni^{2+}	Cu^{2+}	Cd^{2+}	Ni^{2+}
0	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
8	+ + +	nt	nt	+ + +	nt	nt
16	+ + +	nt	nt	+ + +	nt	nt
32	+ +	+ + +	+ + +	+ +	+ + +	+ + +
64	+	+ + +	+ + +	–	+ + +	+ + +
128	–	+ +	+ + +	–	+ +	+ + +
256	– –	–	+ + +	– –	++	+ + +
512	– – –	–	+ + +	– – –	+	+ + +
1024	nt	– – –	– –	nt	– – –	– –
2048	nt	– – –	– –	nt	– – –	– – –

*Key: appearance of cultures on a sliding scale from + + + (healthy: green in *Arabidopsis*, cream in maize) to – – – (unhealthy: yellow in *Arabidopsis*, dark brown in maize); nt = not tested.

Figure legends

Fig. 1: The cascade of ascorbate catabolism in the apoplast.

The reactions labelled '*oxidation*' occur in the presence of ROS, thereby scavenging them. The most effective ROS in each case are indicated; those in brackets react more weakly. Dashed arrows represent non-oxidation reactions, which cannot contribute to ROS scavenging. Adapted from Parsons *et al.* (2011), Parsons and Fry (2012), Dewhirst and Fry (2018) and Dewhirst *et al.* (2020).

Fig. 2: Dose–response curves for the effect of heavy metal ions on growth and cell-aggregate architecture in cell mini-cultures.

(a,c) *Arabidopsis thaliana*; (b,d) *Zea mays*. Mini-cultures (0 d old, i.e. recently diluted into fresh medium) were treated with the heavy metals indicated and cultured for 7 d. (a,b) Growth during 7 d as indicated by increase in packed cell volume (ΔPCV); in some cases the ΔPCV is negative, indicating that the tissue shrank during the culture period. The metal concentrations causing ~50% growth inhibition are indicated by vertical arrows. The absolute PCV at time-zero (by definition ΔPCV = 0) is also shown on the same scale. Each fitted curve is a 4-parameter sigmoidal plot based on all datapoints in the set. (c,d) Susceptibility of the tissue to compaction by centrifugation (SCC), an indication of cell-aggregate architecture, was calculated from settled cell volume (SCV) and PCV.

Fig. 3: Time-course of effect of heavy metal ions on growth of cell mini-cultures.

(a,c) *Arabidopsis thaliana*; (b,d) *Zea mays*. (a,b) Growth in fresh weight; (c,d) growth in packed cell volume. Mini-cultures (0 d old) were treated with the heavy metals indicated and cultured for up to 7 d. The controls received no added heavy metals. Error bars, where larger than the datapoint symbols, indicate range (n=2). The key in (b) applies to all four graphs.

Fig. 4: Time-course of effect of heavy metal ions on endogenous ascorbate.

(a) *Arabidopsis thaliana*; (b) *Zea mays*. Mini-cultures (0 d old) were treated with the heavy metals indicated and cultured for up to 7 d. The control received no heavy metals. Error bars, where larger than the datapoint symbols, indicate range (n=2).

Fig. 5: Short-term effects of heavy metal ions on consumption of exogenous ascorbate.

Loosely capped 0-d-old mini-cultures of (a) *Arabidopsis thaliana* and (b) *Zea mays* were supplemented with the metals as indicated; after 5 min, ascorbate was added to 1 mM and its disappearance from the culture medium during the following 2–4 h incubation was monitored by DCPIP titration. 'No ascorbate', cell-culture with no added metals and no added ascorbate; 'fresh medium', ascorbate incubated in fresh medium with no cells and no added metals; 'no heavy metals', ascorbate incubated with cell-culture but no added metals. The curves were modelled as 2-parameter exponential decay using SigmaPlot; the mean lifetime [τ; the time taken to decrease the ascorbate concentration to 36.8% (=100/e) of its initial value] of the ascorbate is indicated below the *x*-axis in minutes ± SE. Inter-experimental reproducibility can be judged by comparison with a repeat experiment (Fig. S3), via the ± values quoted in Table S1.

Fig. 6: Extracellular L-[1-^{14}C]ascorbate and its metabolites are not taken up by cells nor converted to $^{14}CO_2$.

The experiment was as in Fig. 5, but the added ascorbate contained a trace of [1-^{14}C]ascorbate. At intervals over the following 4–6 h, the concentration of non-volatile ^{14}C remaining in the medium was monitored by scintillation-counting. The control (n=3, with SE shown) received no heavy metals.

Fig. 7: High-voltage paper electrophoresis of extracellular L-[1-^{14}C]ascorbate and its metabolites.

Replicate 25-µl 0-d-old micro-cultures of (a) *Arabidopsis thaliana* and (b) *Zea mays* were supplied with heavy metal ions followed after 5 min by [1-^{14}C]ascorbate to ~1 mM, then killed by addition of formic acid either immediately (0 h) or after 6 h incubation. The metabolites were resolved by high-voltage electrophoresis in a buffer at pH 2.0 and detected by autoradiography. Abbreviations: C, triplicate controls with no heavy-metal pollution; AA mkr, an 'aged' solution of [1-^{14}C]ascorbate showing the positions of many of ascorbate's known catabolites (Dewhirst and Fry, 2018); the 'neutral' spot contains ascorbate and DHA. A non-radioactive marker mixture [MM; containing OG, orange G; ThrR, L-threaric acid (L-tartaric acid); ThrO, L-threonic acid; AA, ascorbic acid; and DHA] was run alongside the radioactive tracks and stained with $AgNO_3$. Each radioactive track contained a trace of internal marker orange G, which is

visible on the unstained electrophoretogram placed as a background to the autoradiogram.

Fig. 1

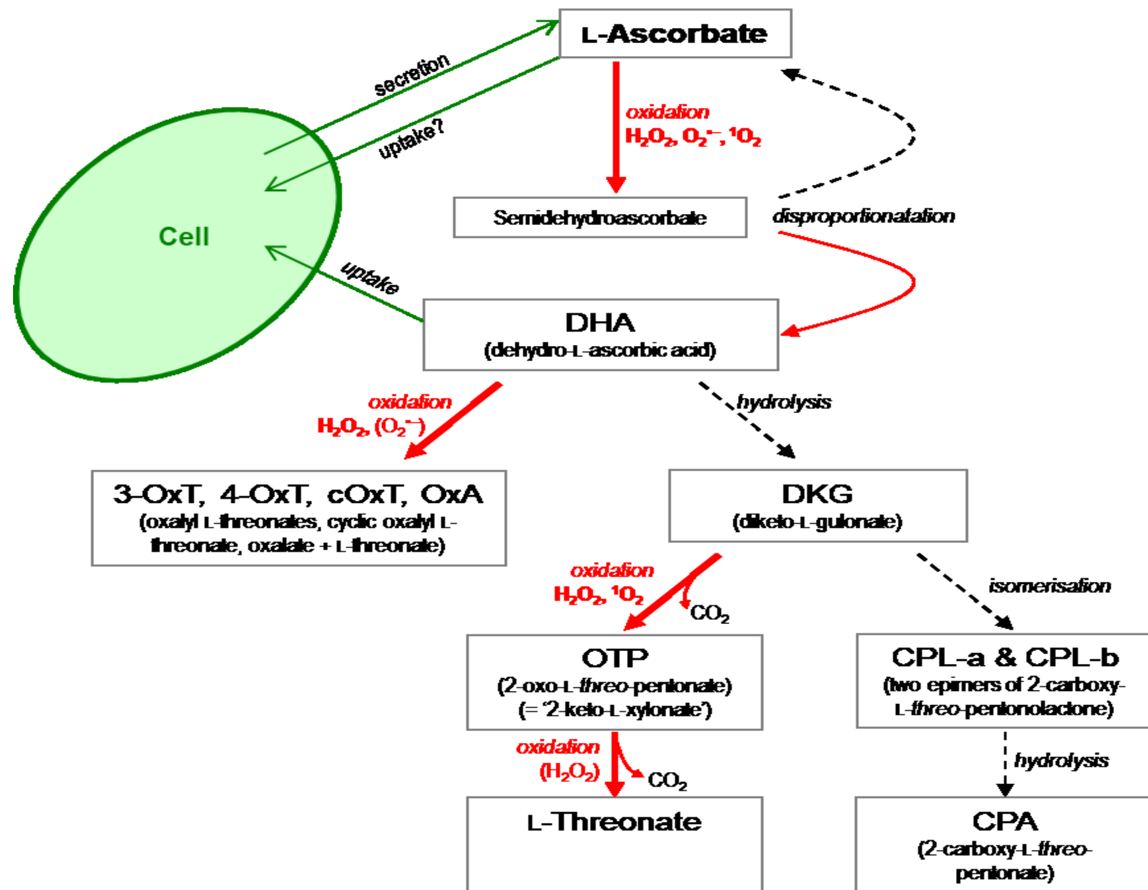

Fig. 2

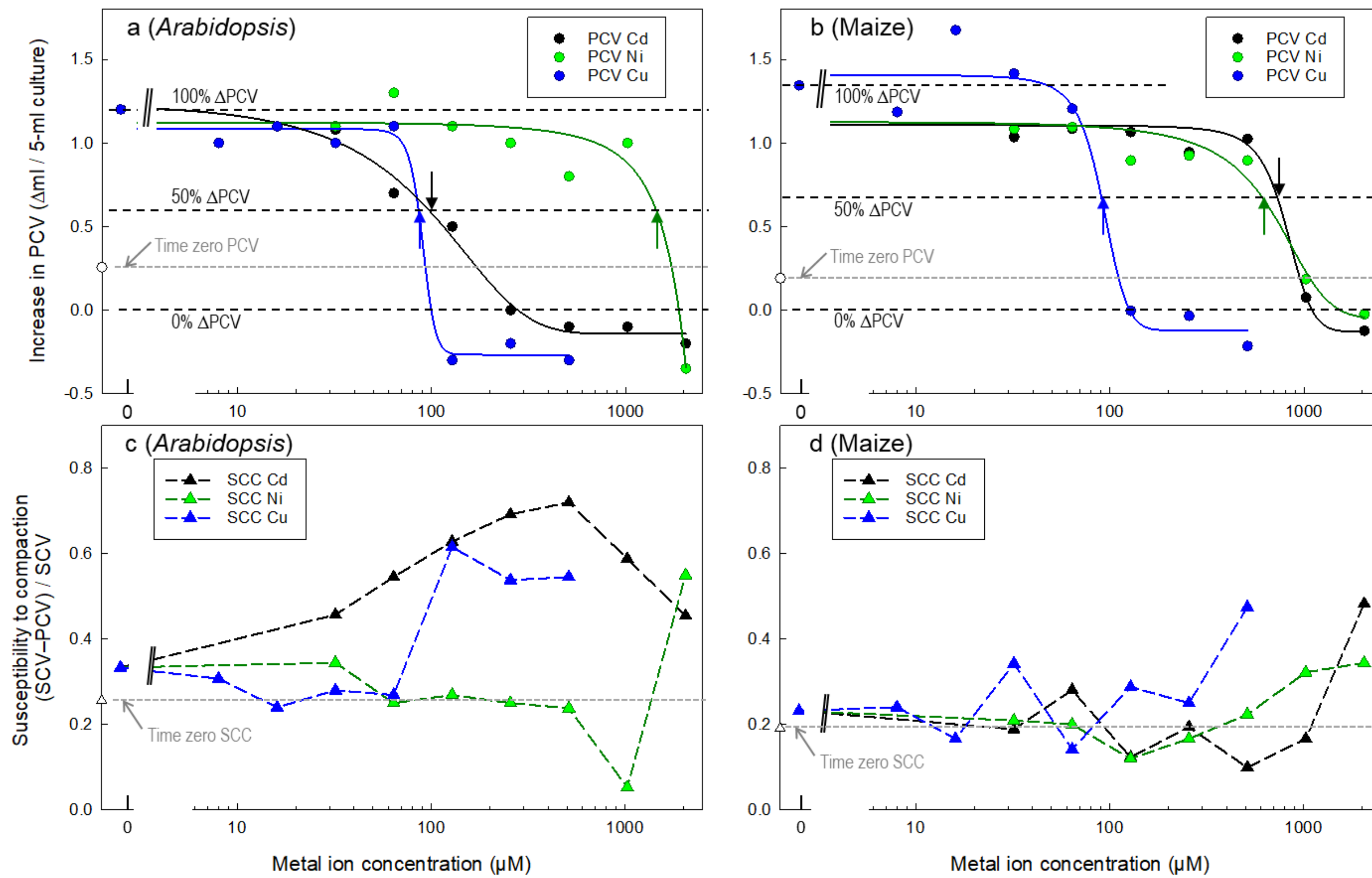

Fig. 3

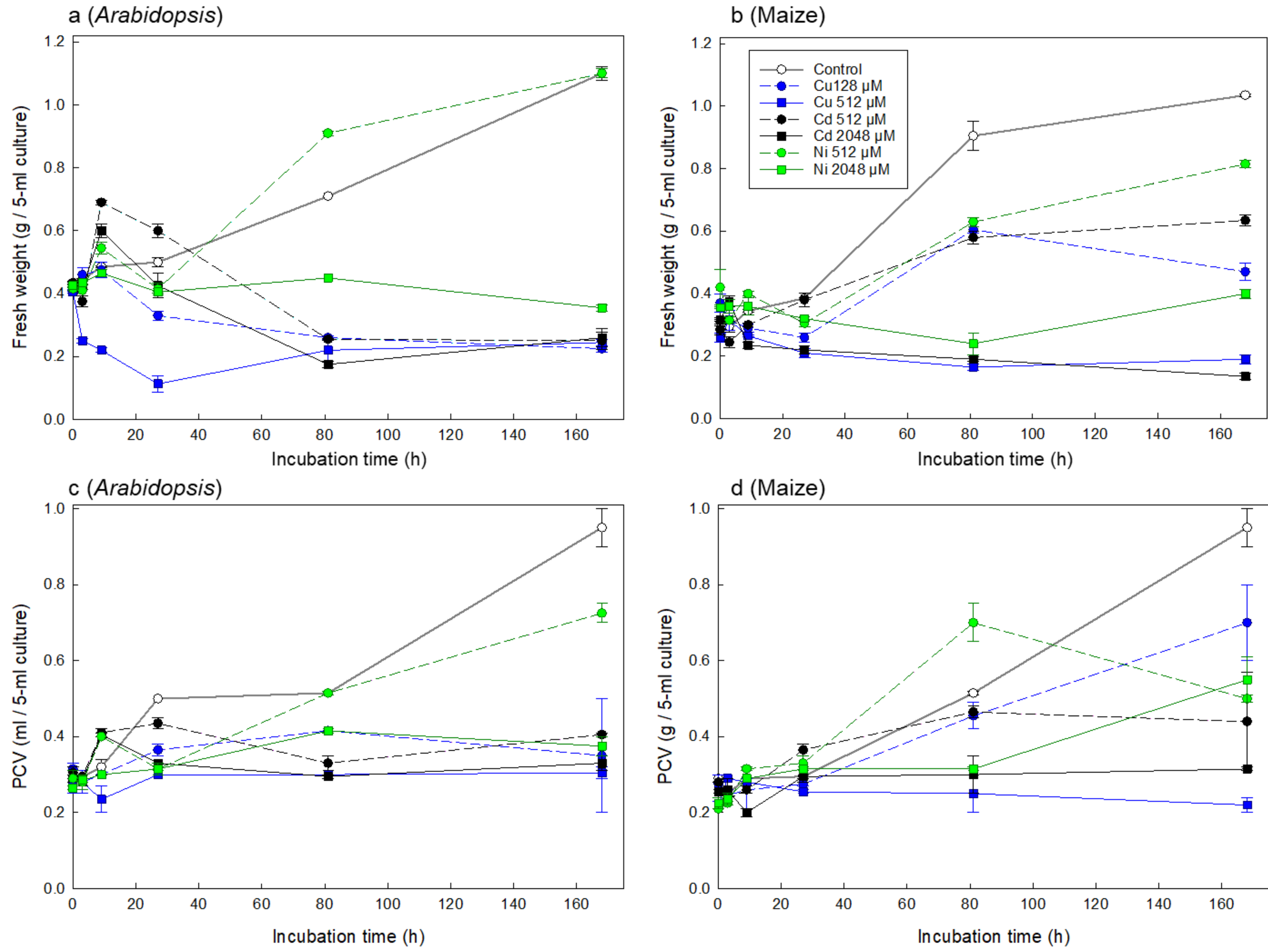

Fig. 4

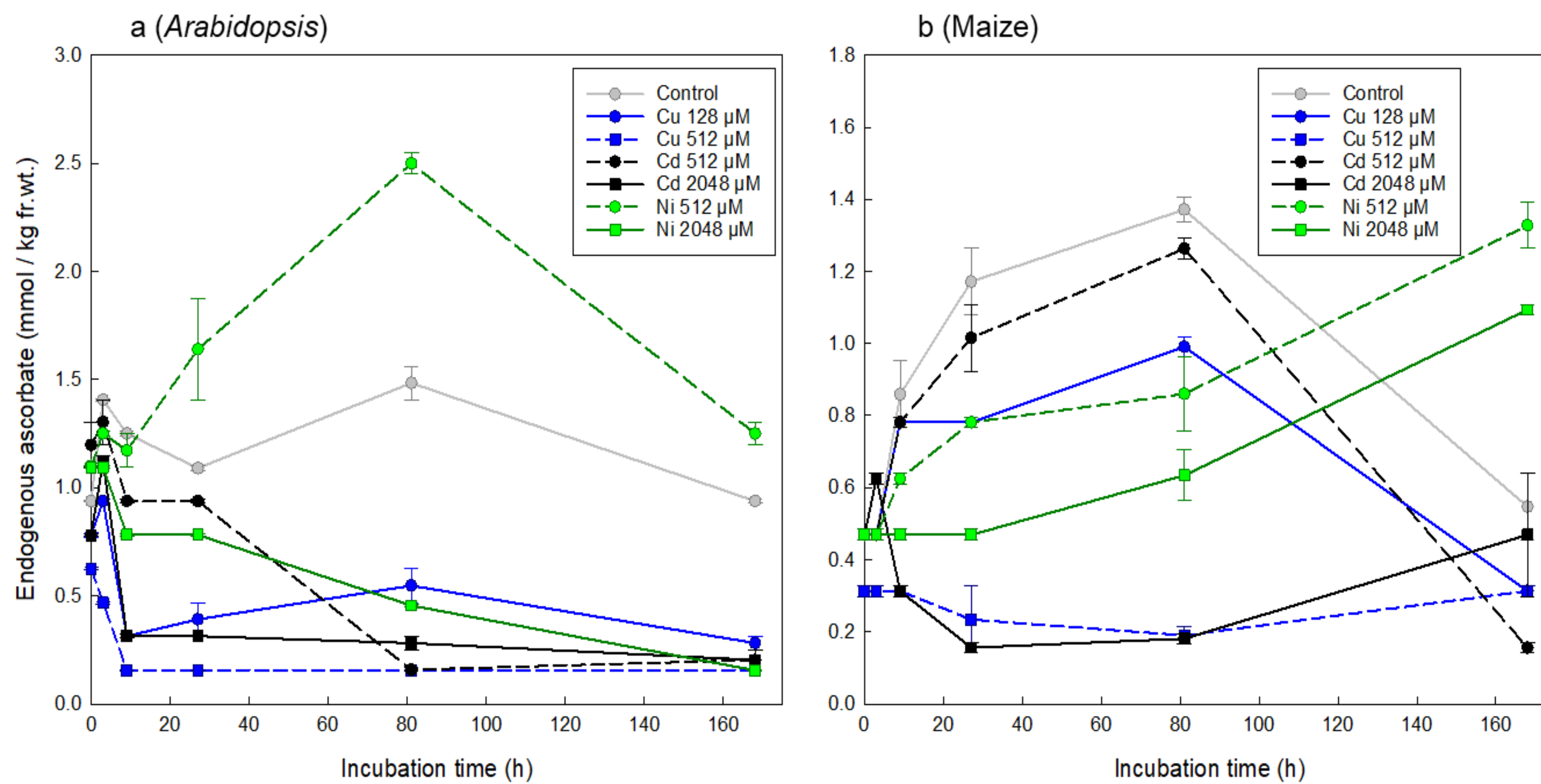

Fig. 5

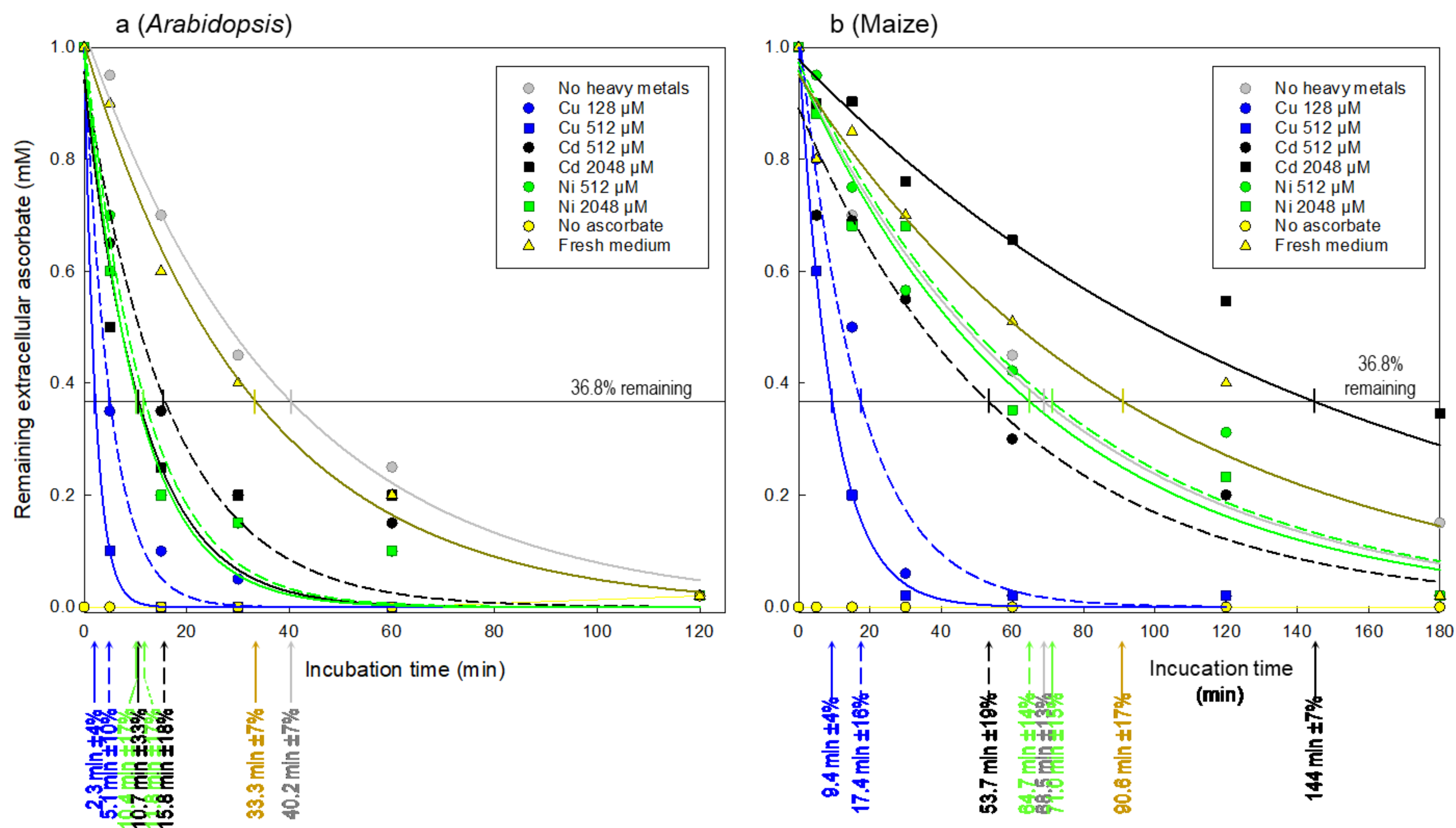

Fig. 6

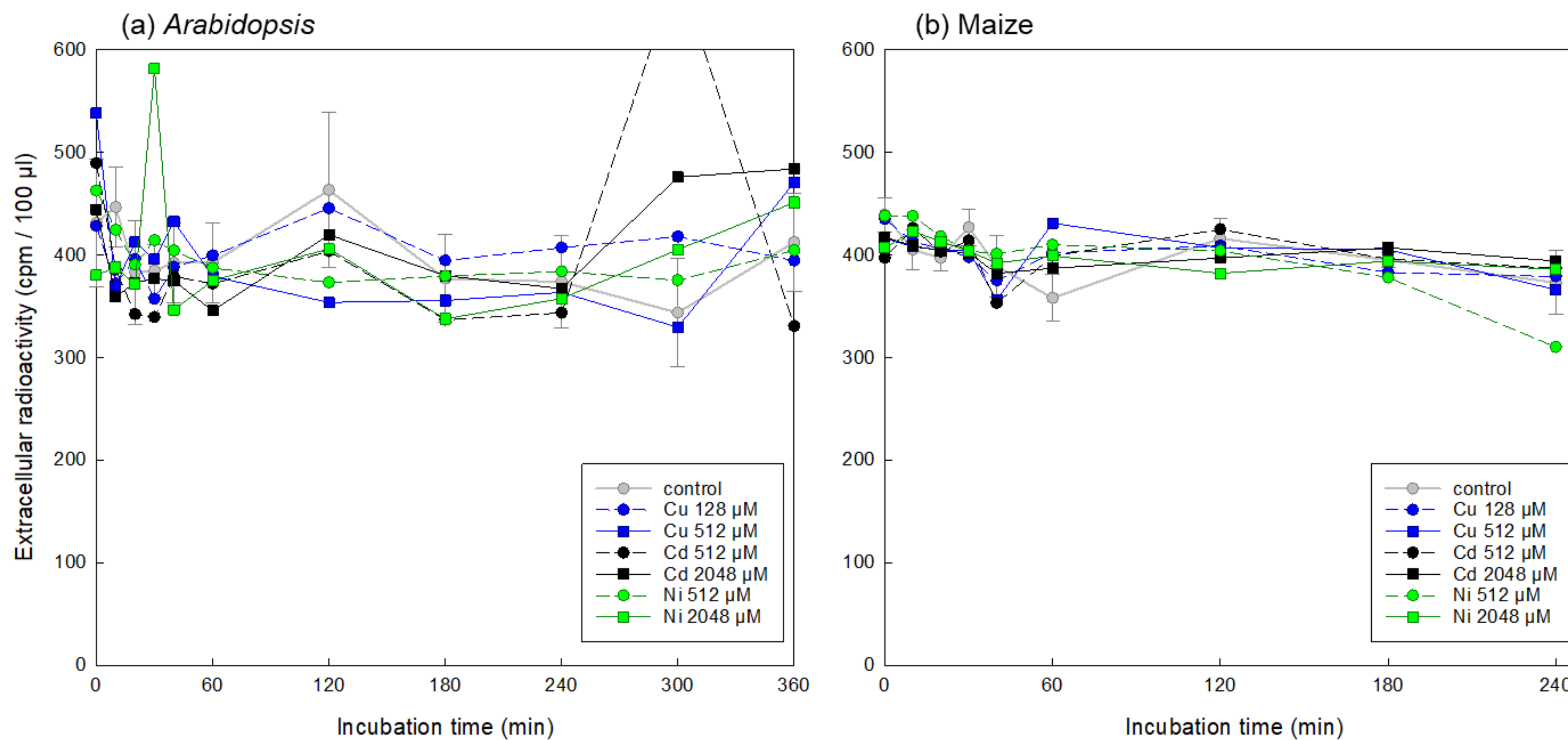

Fig. 7

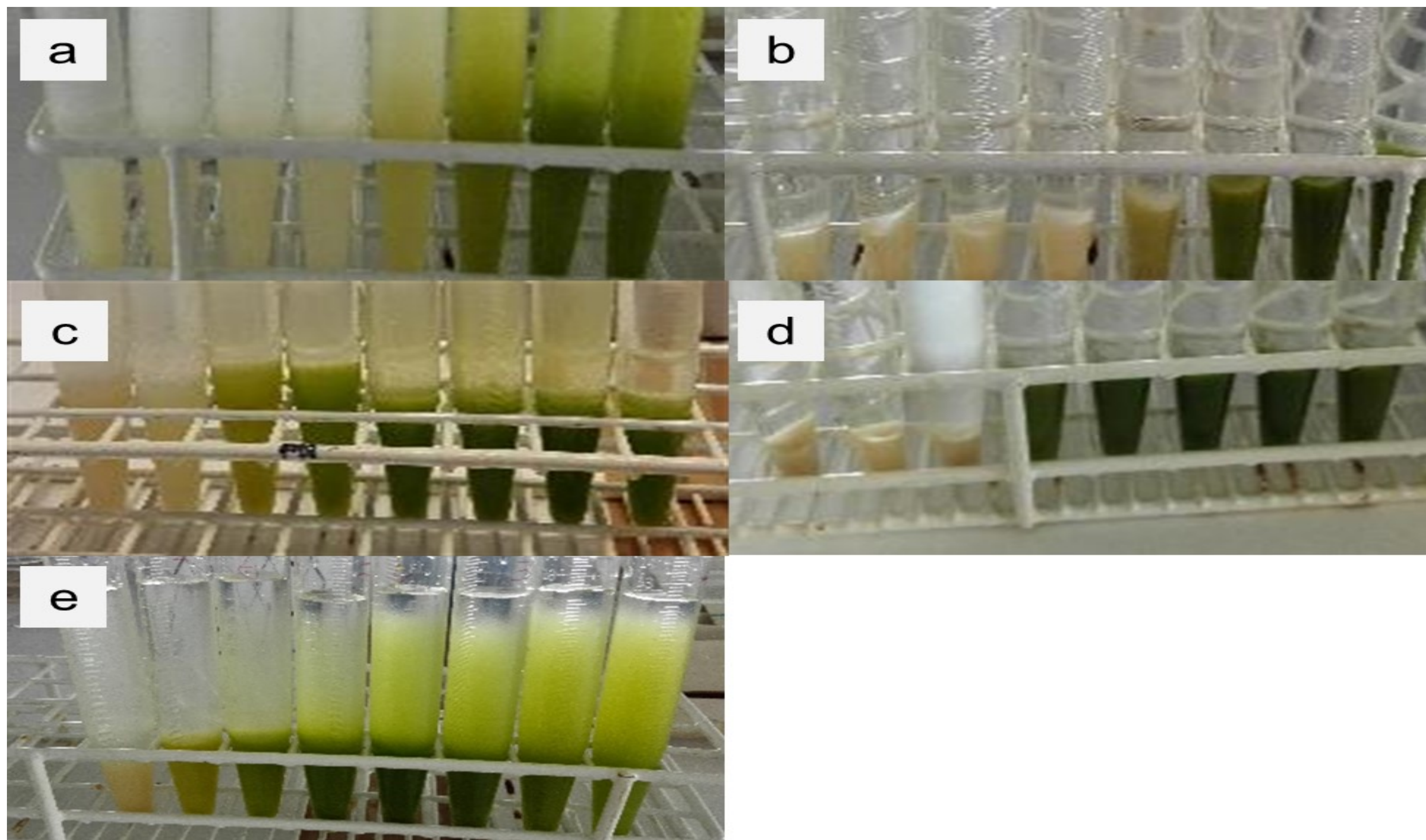

Figure S1: Settled cell volume (SCV) and packed cell volume (PCV) of *Arabidopsis* cultures after 7 days' incubation in the presence of heavy metal ions. SCV (a) and PCV (b) of cultures grown with copper (512, 256, 128, 64, 32, 16, 8, 0 µM, from left to right), SCV (c) and PCV (d) of cultures grown with cadmium (2048, 1024, 512, 256, 128, 64, 32, 0 µM, from left to right), SCV (e) of cultures grown with nickel (2048, 1024, 512, 256, 128, 64, 32, 0 µM, from left to right).

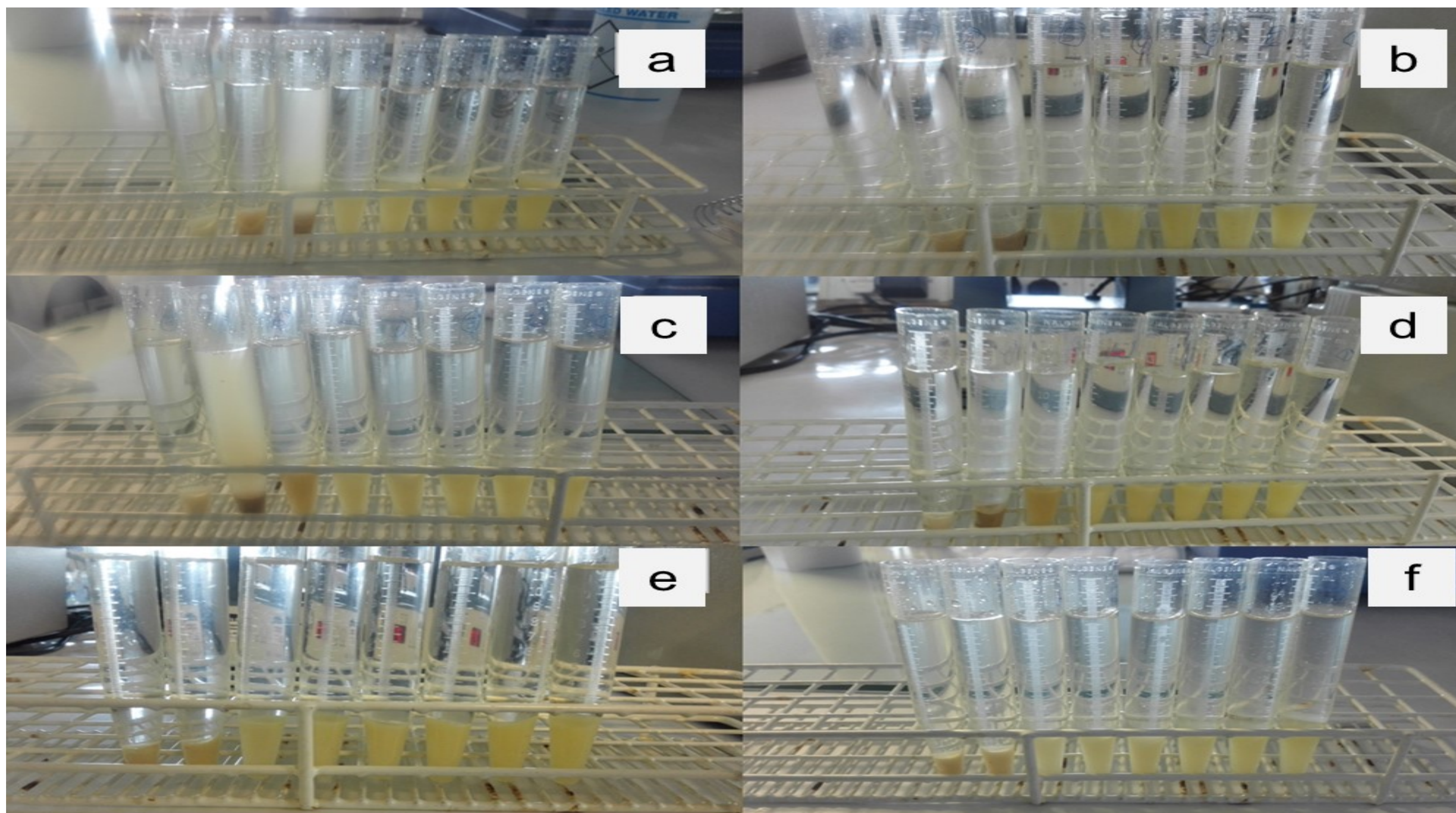

Figure S2: Settled cell volume (SCV) and packed cell volume (PCV) of maize cultures after 7 days' incubation in the presence of heavy metal ions. SCV (a) and PCV (b) of cultures grown with copper (512, 256, 128, 64, 32, 16, 8, 0 µM, from left to right), SCV (c) and PCV (d) of cultures grown with cadmium (2048, 1024, 512, 256, 128, 64, 32, 0 µM, from left to right),SCV (e) and PCV (f) of cultures grown with nickel (2048, 1024, 512, 256, 128, 64, 32, 0 µM, from left to right).

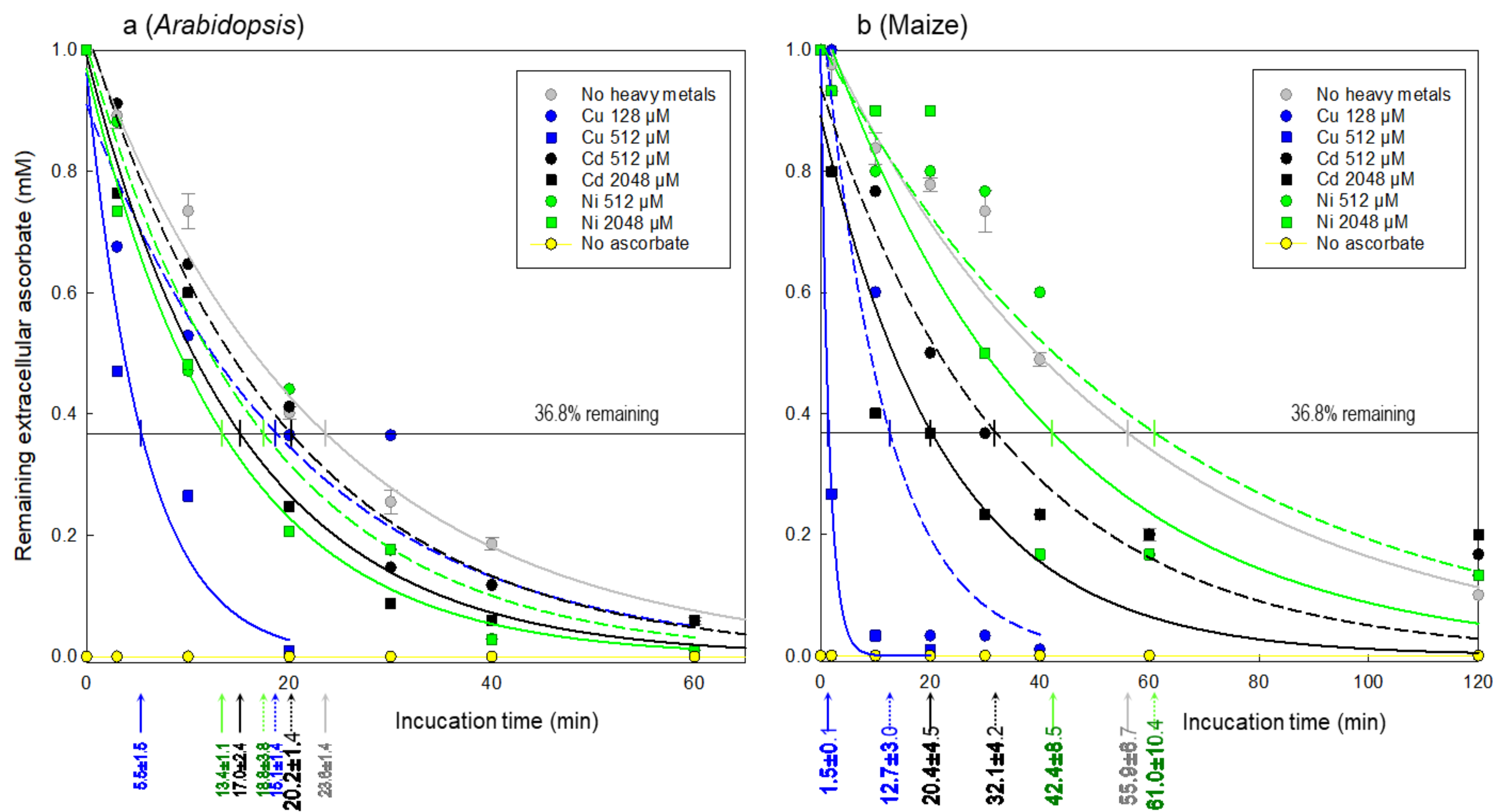

Figure S3: Short-term time-course of the effect of heavy metal ions on consumption of exogenous [^{14}C]ascorbate. Details as in Fig. 5 but the ascorbate was spiked with a trace of [^{14}C]ascorbate.

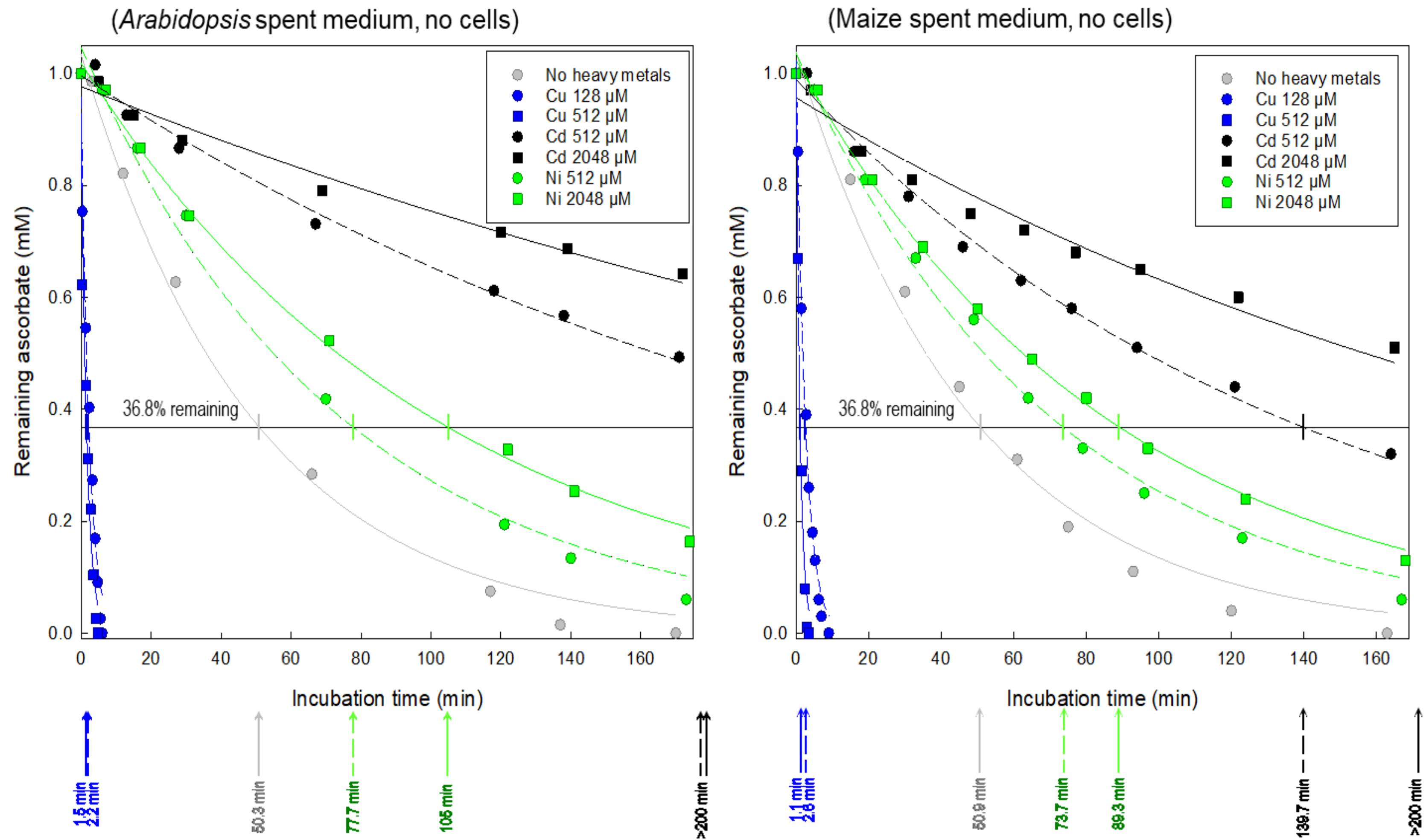

Figure S4: Effect of heavy metal ions on the oxidation of ascorbate in cell-free spent medium
Details as in Fig. 5 but in the absence of cells. Day-zero cultures were prepared (i.e. 1 volume of 1-week-old culture + 2 volumes of fresh medium; final pH 5.5), then the cells were filtered off on Whatman No. 1 paper prior to the addition of heavy metal ions and ascorbate.

Table S1. Time taken for cultures cells to consume extracellular 1 mM ascorbate in the presence of heavy metals.*

Culture	Time (minutes) taken to consume 36.8% of extracellular 1 mM ascorbate in the presence of						
	no heavy metals	Copper		Cadmium		Nickel	
		128 μM	512 μM	512 μM	2048 μM	512 μM	2048 μM
Arabidopsis	32	12	3.9	18	13	14	12
	± 8	± 7	± 1.6	± 2	± 2	± 3	± 2
Maize	62	15	5.5	43	20	66	54
	± 6	± 2	± 3.9	± 11	†	± 5	± 11

*Data are averaged from Fig. 5 and Fig. S1. Data represent mean of two independent experiments ± range.

† n=1.