

# Recovery of cowpea seedling roots from exposure to toxic concentrations of trace metals

F. P. C. Blamey · P. M. Kopittke · J. B. Wehr ·  
N. W. Menzies

Received: 25 August 2010 / Accepted: 8 November 2010 / Published online: 23 December 2010  
© Springer Science+Business Media B.V. 2010

**Abstract** Rhizotoxic effects of many trace metals are known, but there is little information on recovery after exposure. Roots of 3-d-old cowpea (*Vigna unguiculata* (L.) Walp. cv. Caloona) seedlings were grown for 4 or 12 h in solutions of 960  $\mu\text{M}$  Ca and 5  $\mu\text{M}$  B at two concentrations (which reduce growth by 50 or 85%) of nine trace metals that rupture the outer layers of roots. Measured concentrations were 34 or 160  $\mu\text{M}$  Al, 0.6 or 1.6  $\mu\text{M}$  Cu, 2.2 or 8.5  $\mu\text{M}$  Ga, 2.3 or 12  $\mu\text{M}$  Gd, 0.8 or 1.9  $\mu\text{M}$  Hg, 1.0 or 26  $\mu\text{M}$  In, 2.4 or 7.3  $\mu\text{M}$  La, 1.8 or 3.8  $\mu\text{M}$  Ru, and 1.3 or 8.6  $\mu\text{M}$  Sc. Roots were rinsed, transferred to solutions free of trace metals, and regrowth monitored for up to 48 h. Recovery from exposure to Hg occurred within 4 h, but regrowth was delayed for  $\geq 12$  h with Al, Ga, or Ru. There was poor regrowth after 4 or 12 h exposure to Cu, Gd, In, La, or Sc. Roots recovered after being grown for 12 to 48 h in 170  $\mu\text{M}$  Al, 5.1  $\mu\text{M}$  Ga, 2.0  $\mu\text{M}$  Hg, or 1.4  $\mu\text{M}$  Ru, but the extent of recovery was reduced with longer

exposure time. Microscopy showed marked differences in symptoms on roots recovering from exposure to the various trace metals. Differences in (i) concentrations that are toxic, (ii) ability of roots to recover, (iii) time for recovery to occur, and (iv) symptoms that develop, suggest that each trace metal has a unique combination of rhizotoxic effects.

**Keywords** Cowpea · Aluminium · Copper · Gallium · Gadolinium · Indium · Lanthanum · Mercury · Ruthenium · Scandium · Toxicity

## Abbreviations

FIMS-AAS	flow injection mercury system-atomic absorption spectroscopy
ICP-OES/MS	inductively coupled plasma-optical emission spectrometry/mass spectrometry
RER	root elongation rate (mm/h)

Communicated by Juan Barcelo.

F. P. C. Blamey (✉) · P. M. Kopittke · J. B. Wehr ·  
N. W. Menzies  
School of Land, Crop and Food Sciences,  
The University of Queensland,  
St. Lucia, Queensland, Australia 4072  
e-mail: p.blamey@uq.edu.au

F. P. C. Blamey · P. M. Kopittke · N. W. Menzies  
Cooperative Research Centre for Contamination  
Assessment and Remediation of the Environment  
(CRC-CARE), The University of Queensland,  
St. Lucia, Queensland, Australia 4072

## Introduction

The levels of trace metals in many instances are at concentrations that are toxic to plants and animals. Examples include elevated levels in soils of soluble aluminium (Al) (von Uexküll and Mutert 1995) and cadmium (Cd) (McLaughlin et al. 1996) through agricultural practices; cobalt (Co), copper (Cu), nickel (Ni), and zinc (Zn) through mining; and lead (Pb) through the use of batteries and leaded fuel. Recently,

many uncommon trace metals, including gallium (Ga), gadolinium (Gd), mercury (Hg), indium (In), and ruthenium (Ru), have been used in increasing quantities in the electrical, electronics, computer and medical industries (Babula et al. 2008; Patra et al. 2004). There are increasing concerns as to their possible toxicity to plants and animals.

Trace metals may be toxic through inducing oxidative stress, bonding with proteins or other compounds, and disruption of cell division (Babula et al. 2008; Patra et al. 2004). There are many measures of trace metal intoxication, all of which help to determine the underlying causes of toxicity. The adverse effects on growth can be determined using the threshold of toxicity (Taylor et al. 1991) and the timeframe in which toxic effects become evident. Root growth inhibition occurs in  $\leq 1$  h with Al in mungbean (*Vigna radiata* L.) (Blamey et al. 2004) or after  $\geq 2$  h with many other trace metals in cowpea (*Vigna unguiculata* (L.) Walp.) (Kopittke et al. 2008; Kopittke et al. 2009). The appearance, kinetics, location, and magnitude of symptoms that develop are useful also, and additional evidence may be provided by recovery from exposure to toxic concentrations of trace metals.

Some trace metals have severe rhizotoxic effects at  $< 1.0$ – $30$   $\mu\text{M}$ , while others, including strontium (Sr) and manganese (Mn), are rhizotoxic only at higher concentrations. Given the extent of acid soils (von Uexküll and Mutert 1995), many studies have evaluated the adverse effects of soluble trivalent Al on root growth. The toxic effects of the trivalent ions, Ga, Gd, lanthanum (La) and scandium (Sc), have been used as Al analogues to help elucidate the rhizotoxic effect of Al (Bennet and Breen 1991; Clarkson 1965; Hou et al. 2010; Reid et al. 1996).

The rhizotoxic effects of these and other trace metals have been studied in their own right also (Kopittke et al. 2008; Kopittke et al. 2009), but few studies have assessed the recovery of roots after intoxication. Such studies may elucidate mechanisms of detoxification (e.g. metal sequestration, repair of damage by replacement of inactivated enzymes) and may shed light on the physiological mechanisms of toxicity.

Bennet and Breen (1991) found that maize (*Zea mays* L.) roots regrow in a subsequent 8 d period after growing for 2–48 h in solution containing 185  $\mu\text{M}$  Al (though nearly one half of the roots failed to regenerate the primary apex after 48 h in Al solution). It was concluded that exposure to Al stimulated root regrowth

after 6 h in Al solution (root elongation rate (RER)= 1.4 mm/h). This conclusion is probably incorrect since it was based on a RER of only 0.6 mm/h in the control treatment; maize has a RER of 1.0–2.0 mm/h under non-limiting conditions (Kollmeier et al. 2000; Sivaguru et al. 2006). Wagatsuma et al. (2005) found that the roots of two triticale (*Triticosecale* sp. Wittmark) lines differing in Al-tolerance recovered after growing for 1 or 24 h in 20  $\mu\text{M}$  Al when transferred to 0.2 mM Ca solution. Plasma membrane permeability of root-tip cells in the Al-sensitive line increased after the recovery period. Recovery from Al stress in bean (*Phaseolus vulgaris* L.) was associated with a decrease of Al in the root tip, especially in the transition zone (Rangel et al. 2007). Scott et al. (2008) used root regrowth after 24 h exposure to Al in solution to select for Al tolerance among 16 *Medicago* genotypes. Motoda et al. (2010) demonstrated regrowth in Al-intoxicated pea (*Pisum sativum* (L.) roots, concluding that “only the apical region of an Al-treated root can resume elongation in Al-free solution”. In a study which focussed on the effects of Al on the actin cytoskeleton, roots of an Al-sensitive maize line recovered after 6 h in Al-free solution (Amenós et al. 2009). The results of these studies show that plant roots are able to regrow after exposure to Al, but further information is needed on short-term effects and whether or not roots recover from exposure to other trace metals.

Solution culture experiments were conducted to test the hypothesis that root recovery after exposure differs among trace metals, with higher concentration and longer duration having more detrimental effects. The study focused on divalent and trivalent trace metals which rupture the rhizodermis and outer cortex of cowpea roots at a low threshold of toxicity ( $< \text{ca. } 30$   $\mu\text{M}$ ) (Kopittke et al. 2008; Kopittke et al. 2009). The overall aim of the study was to quantify the similarities and differences among trace metal effects on root growth and recovery to gain further understanding of the underlying mechanisms of toxicity.

## Materials and methods

### General experimental procedures

Cowpea (*Vigna unguiculata* (L.) Walp.) (cv. Caloona) seeds were germinated in rolled paper towels in tap water, and seven 3-d-old seedlings were transplanted

into holes in a Perspex strip and grown for 12–16 h in 650 mL aerated solutions of ca. 1000  $\mu\text{M}$   $\text{CaCl}_2$  and 5  $\mu\text{M}$   $\text{H}_3\text{BO}_3$  (pH 5.3) (Kopittke et al. 2008). The seedlings were transferred to aerated solutions of Ca and B (as above) plus one trace metal. Roots were grown for specified periods in these solutions before rinsing twice in Ca and B solution (as above) and transfer to recovery solutions of ca. 1000  $\mu\text{M}$   $\text{CaCl}_2$  and 5  $\mu\text{M}$   $\text{H}_3\text{BO}_3$  (pH 5.3). Ambient temperature was maintained at 25°C throughout.

Solution pH was measured and samples of trace metal solutions taken at the beginning (i.e. immediately after mixing through aeration) and at the end of the exposure periods, filtered to 0.22  $\mu\text{m}$  (Millipore, Millex-GS, SLGS033SS), acidified to pH < 2.0 using 20  $\mu\text{L}$  of concentrated HCl (32%), and refrigerated (4°C) before analysis. Analysis was by inductively coupled plasma-optical emission spectrometry (ICP-OES) for Ca, Ru, and Sc, inductively coupled plasma-mass spectrometry (ICP-MS) for Ga, Gd, and In, and flow injection mercury system-atomic absorption spectroscopy (FIMS-AAS) for Hg. Unless otherwise stated, all metal concentrations listed are the means of the values measured at the beginning and end of the growth period in the trace metal solutions. To gain further understanding of trace metal concentrations in solution, Phreeqc1 2.13.04 with the Minteq database (Parkhurst 2007) was used to estimate solution speciation using the mean measured solution pH and concentrations of Ca and each trace metal at the beginning of the experiments (i.e. immediately after mixing). Additional equilibrium constants were added to the Minteq database (Kopittke et al. 2009) except for Ru for which no values were found.

#### Preliminary experiments

A broad range in concentrations (0.1–200  $\mu\text{M}$ ) of trace metals in solutions with ca. 1000  $\mu\text{M}$  Ca and 5  $\mu\text{M}$  B was evaluated to establish the concentrations that reduce root growth and to determine whether or not ruptures develop in the outer layers of the roots. These studies demonstrated that there are large differences in the threshold concentration and in the decrease in root growth with further increase in trace metal concentration. The time taken for root growth to decrease differed also (e.g. a 50% reduction in RER was evident after 4 h growth with 1.0  $\mu\text{M}$  Hg but only after 12 h with 3.6  $\mu\text{M}$  La). There was a clear

reduction in root growth after 48 h exposure, and concentrations causing a 50 and 85% decrease in RER at this time were chosen as those to which cowpea roots were to be exposed.

There was no evidence in these preliminary experiments of any benefit to root growth of any of the trace metals studied despite the wide range in concentrations used (see Buckingham et al. (1999) and Babula et al. (2008) for reviews of reported beneficial effects of lanthanoids on plant growth).

#### Experiment 1

Based upon the results of the preliminary experiments, Experiment 1 investigated the recovery of roots from exposure to nine trace metals, Al, Cu, Ga, Gd, Hg, In, La, Ru, or Sc, that rupture the outer layers of roots (Kopittke et al. 2008; Kopittke et al. 2009). A total of 40 treatments was established, consisting of two exposure periods (4 or 12 h), ten metals (control plus nine trace metals), and two exposure concentrations (those expected to result in a 50 or 85% reduction in RER after 48 h) (Table 1). Solution pH was measured but not adjusted in any treatment. The treatments were replicated twice over time, with each experimental unit containing seven seedlings (the average of these forming one replicate), giving a total of 80 experimental units and 560 roots.

Seedlings were grown for 16 h in ca. 1000  $\mu\text{M}$   $\text{CaCl}_2$  and 5  $\mu\text{M}$   $\text{H}_3\text{BO}_3$  (pH 5.3) solutions. Thereafter, the roots were transferred to the appropriate metal solution for either 4 or 12 h before being rinsed twice and transferred to recovery solutions of ca. 1000  $\mu\text{M}$   $\text{CaCl}_2$  and 5  $\mu\text{M}$   $\text{H}_3\text{BO}_3$ . Digital images were captured for each treatment at the start and end of the exposure periods, and at 4, 8, 12, 24, 36, and 48 h during the recovery period. Root length was measured (Kopittke et al. 2008), allowing the calculation of individual root elongation rate (RER). Thus, Experiment 1 included a total of 4480 measurements of root length.

At the end of the experimental period (i.e. after 48 h recovery), the distal 50 mm section of each root was placed in a 10% ethanol solution and refrigerated (4°C) for up to 1 d. These unstained sections were examined under a dissecting light microscope, and digital images captured. To further examine symptom development, additional beakers were set up with Ca and B (as above) and the higher concentration of each

**Table 1** Nominal and mean measured Ca and trace metal concentrations and pH ( $\pm$  SD) in solutions in which cowpea roots were grown for 4 or 12 h prior to transfer to recovery solutions (Experiment 1). The nominal concentrations of trace metals are those determined in the preliminary experiments to reduce root elongation by 50 or 85% during a 48 h exposure period. Solutions were sampled at the beginning and end of the two exposure periods. The Ca concentration was measured in solutions of all control and trace metal treatments ( $n=40$ );  $n=4$  for all other values

Metal	Concentration ( $\mu\text{M}$ )			Solution pH	
	Nominal	Measured (4 h)	Measured (12 h)	(4 h)	(12 h)
Ca	1000	960 $\pm$ 40	960 $\pm$ 40	5.33 $\pm$ 0.04	5.40 $\pm$ 0.11
Al	30	33 $\pm$ 1	35 $\pm$ 2	4.83 $\pm$ 0.01	4.80 $\pm$ 0.03
Al	150	160 $\pm$ 4	170 $\pm$ 7	4.52 $\pm$ 0.02	4.49 $\pm$ 0.04
Cu	0.5	0.6 $\pm$ 0.1	0.5 $\pm$ 0.0	5.30 $\pm$ 0.15	5.39 $\pm$ 0.15
Cu	1.5	1.6 $\pm$ 0.2	1.5 $\pm$ 0.1	5.35 $\pm$ 0.07	5.43 $\pm$ 0.09
Ga	30	1.7 $\pm$ 0.3	2.6 $\pm$ 1.3	4.44 $\pm$ 0.03	4.40 $\pm$ 0.07
Ga	60	7.1 $\pm$ 1.3	9.8 $\pm$ 3.8	4.14 $\pm$ 0.02	4.10 $\pm$ 0.06
Gd	3	2.2 $\pm$ 0.2	2.3 $\pm$ 0.3	5.29 $\pm$ 0.07	5.34 $\pm$ 0.11
Gd	15	12 $\pm$ 0.4	12 $\pm$ 0.8	5.24 $\pm$ 0.03	5.25 $\pm$ 0.03
Hg	1	0.7 $\pm$ 0.1	0.8 $\pm$ 0.1	5.62 $\pm$ 0.04	5.54 $\pm$ 0.10
Hg	2.5	1.8 $\pm$ 0.1	1.9 $\pm$ 0.2	5.73 $\pm$ 0.07	5.64 $\pm$ 0.16
In	3	0.6 $\pm$ 0.1	1.3 $\pm$ 0.9	5.14 $\pm$ 0.04	5.17 $\pm$ 0.07
In	40	23 $\pm$ 2	29 $\pm$ 9	5.35 $\pm$ 0.01	4.43 $\pm$ 0.10
La	2	2.4 $\pm$ 0.1	2.4 $\pm$ 0.2	5.35 $\pm$ 0.01	5.36 $\pm$ 0.10
La	6	7.1 $\pm$ 0.3	7.4 $\pm$ 0.5	5.26 $\pm$ 0.02	5.34 $\pm$ 0.09
Ru	10	1.4 $\pm$ 0.4	2.1 $\pm$ 1.0	4.71 $\pm$ 0.07	4.72 $\pm$ 0.05
Ru	20	3.6 $\pm$ 0.8	4.0 $\pm$ 1.1	4.45 $\pm$ 0.03	4.44 $\pm$ 0.05
Sc	2	1.2 $\pm$ 0.1	1.3 $\pm$ 0.3	5.26 $\pm$ 0.06	5.28 $\pm$ 0.06
Sc	10	8.4 $\pm$ 0.3	8.7 $\pm$ 0.7	4.99 $\pm$ 0.00	5.00 $\pm$ 0.01

trace metal, seedlings grown, and roots harvested at the end of a 12 h exposure period and after 2, 4, 8, 12, 24, 36, and 48 h recovery. Micrographs of these roots were captured as before.

## Experiment 2

This solution culture experiment focussed on the four trace metals (viz. Al, Ga, Hg, and Ru) from which roots were found to recover well in Experiment 1. There were 20 treatments: four exposure periods (12, 24, 36, or 48 h), five metals (control plus four trace metals), and one exposure concentration that was expected to produce an 85% reduction in RER during 48 h exposure.

The same procedure was used as in Experiment 1, with batches of seeds germinated at 12 h intervals, each 3 d before transplanting into ca. 1000  $\mu\text{M}$   $\text{CaCl}_2$  and 5  $\mu\text{M}$   $\text{H}_3\text{BO}_3$  (pH 5.3) solutions in which they were grown for 12 h. Thereafter, the roots were grown in the metal treatments as in Experiment 1 (Table 2). Roots were grown for 12, 24, 36, or 48 h in these solutions before transfer after rinsing to recovery

solutions in which they were grown for 48 h. Solutions were sampled at the beginning and end of each exposure period for analysis as in Experiment 1. Digital images for root length measurement were captured at intervals of 12 h during the exposure period, at the end of the exposure period, and after 4, 8, 12, 24, 36, and 48 h recovery. This resulted in a total of 2660 measurements of root length.

## Results

### Solution composition

Measurements of Ca, Al, Cu, Gd, Hg, and La in filtered solutions from Experiment 1 showed that the concentrations were similar to the nominal concentrations over the 4 and 12 h exposure periods (Table 1), with Ca being 4% lower, Al 8–17% higher, and Cu and La up to 20% higher than intended. Measured concentrations of Gd and Hg were ca. 30% lower than the nominal concentrations, with that of Sc 10–40% lower. These differences, however, were

**Table 2** Nominal and mean measured Ca and trace metal concentrations and pH ( $\pm$  SD) in solutions in which cowpea roots were grown for 12 to 48 h prior to transfer to recovery solutions (Experiment 2). The nominal concentrations of trace metals are those determined in the preliminary experiment to

reduce root elongation by 85% during a 48 h exposure period. Solutions were sampled at the beginning and end of each exposure period. The Ca concentration was measured in solutions of all control and trace metal treatments ( $n=60$ );  $n=4$  for all other values

Metal	Nominal concentration ( $\mu\text{M}$ )	Exposure period (h)							
		Measured concentration ( $\mu\text{M}$ )				Solution pH			
		12	24	36	48	12	24	36	48
Ca	1000	940 $\pm$ 40				5.12 $\pm$ 0.11	5.22 $\pm$ 0.23	5.08 $\pm$ 0.23	5.13 $\pm$ 0.29
Al	150	170 $\pm$ 8	170 $\pm$ 6	170 $\pm$ 8	170 $\pm$ 4	4.37 $\pm$ 0.02	4.39 $\pm$ 0.04	4.36 $\pm$ 0.02	4.34 $\pm$ 0.01
Ga	60	4.6 $\pm$ 2.7	9.2 $\pm$ 3.0	3.2 $\pm$ 1.6	3.4 $\pm$ 1.3	3.97 $\pm$ 0.02	3.97 $\pm$ 0.02	4.03 $\pm$ 0.07	4.01 $\pm$ 0.09
Hg	2.5	1.2 $\pm$ 0.5	1.9 $\pm$ 0.3	2.3 $\pm$ 0.3	2.5 $\pm$ 0.1	5.73 $\pm$ 0.07	5.58 $\pm$ 0.23	5.71 $\pm$ 0.12	5.57 $\pm$ 0.26
Ru	20	1.3 $\pm$ 0.7	2.7 $\pm$ 0.9	0.5 $\pm$ 0.1	1.1 $\pm$ 1.0	4.32 $\pm$ 0.05	4.30 $\pm$ 0.06	4.34 $\pm$ 0.08	4.33 $\pm$ 0.08

small relative to those of Ga, In, and Ru. The measured Ga concentration in solution was only 6–16% of the nominal concentration; corresponding values were 20–73% for In and 14–21% for Ru. With these trace metals, there was an initial, marked decrease in concentration immediately after mixing followed by a more gradual decrease over the following 8 and 12 h (data not presented), consistent with previous studies (Kopittke et al. 2009). Thermodynamic modelling with Phreeqc1 2.13.04 (Parkhurst 2007) indicated that Al, Cu, Gd, and La were present almost exclusively as the free ion ( $\text{Al}^{3+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Gd}^{3+}$ , or  $\text{La}^{3+}$ ). However, solutions of Ga, Gd, In, or Sc were saturated with  $\text{Ga}(\text{OH})_3(\text{s})$ ,  $\text{Gd}(\text{OH})_3(\text{s})$ ,  $\text{In}(\text{OH})_3(\text{s})$ , or  $\text{Sc}(\text{OH})_3(\text{s})$  (no solubility data were available for  $\text{Ru}(\text{OH})_3(\text{s})$ , but a black precipitate was visible in solution and adhering to the roots and beaker).

Solutions in the control treatments were at pH 5.3, a similar value being evident in those solutions to which Cu, Gd, In, Ru, or Sc were added (Table 1). Addition of Al, Ga, Ru, and Sc resulted in a slight decrease in solution pH to values  $\geq$ pH 4.4. Only in one instance (viz. the higher Ga concentration) was there a decline to ca. pH 4.1 which may have decreased cowpea root elongation through  $\text{H}^+$  toxicity (unpublished data). Addition of Hg slightly increased pH (Table 1).

Solution composition in Experiment 2 (Table 2) was similar to that in Experiment 1, with modelling producing similar results.

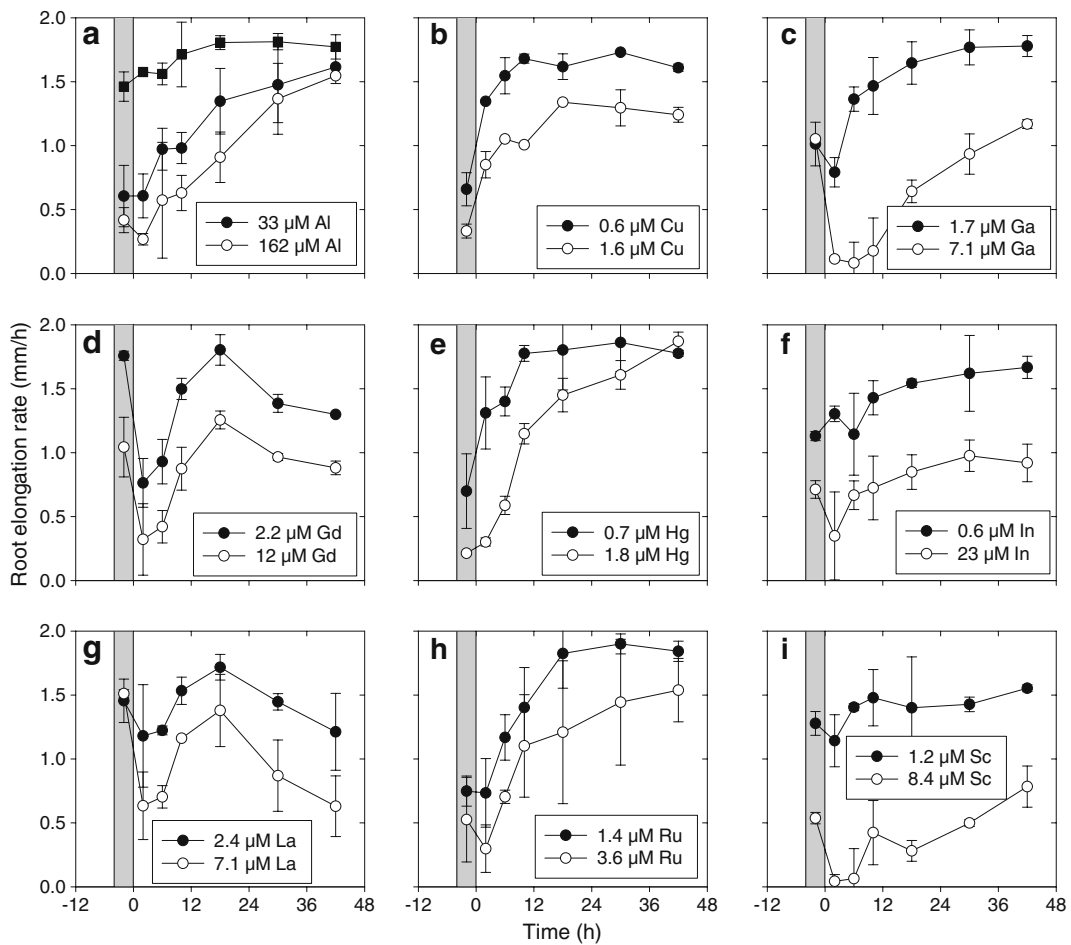
#### Root growth during exposure to trace metals

There was good root growth in the control treatments in Experiment 1, mean RER over the experimental

periods (52 and 60 h) being  $1.71\pm 0.18$  mm/h ( $n=56$ ). During the 4 h period of exposure, there were marked differences among the trace metals in their detrimental effects on RER (Fig. 1). The lower concentration of each trace metal was chosen to cause a 50% decrease in RER when exposed for 48 h, this largely being achieved at measured concentrations of  $33 \mu\text{M}$  Al,  $0.6 \mu\text{M}$  Cu,  $0.7 \mu\text{M}$  Hg, and  $1.4 \mu\text{M}$  Ru. Exposure for 4 h to  $1.7 \mu\text{M}$  Ga and  $1.2 \mu\text{M}$  Sc was less detrimental while  $2.2 \mu\text{M}$  Gd or  $2.4 \mu\text{M}$  La had no noticeable adverse effect during the exposure period. Exposure of roots for 4 h to the higher concentration of each trace metal (chosen to reduce RER by 85%) decreased RER by 85% with  $1.8 \mu\text{M}$  Hg and by 60–75% with  $160 \mu\text{M}$  Al,  $1.6 \mu\text{M}$  Cu,  $3.6 \mu\text{M}$  Ru, and  $8.4 \mu\text{M}$  Sc. The effects of  $7.1 \mu\text{M}$  Ga and  $11.7 \mu\text{M}$  Gd were less severe, with RER reduced by 25–30%. There was no detrimental effect of  $7.1 \mu\text{M}$  La on root growth during the exposure period of 4 h.

The longer exposure time of 12 h resulted in RER close to that envisaged (Fig. 2). Overall, the lower concentration of each trace metal decreased RER to  $54\pm 18\%$  of the control, with  $1.3 \mu\text{M}$  In and  $2.4 \mu\text{M}$  La being the least detrimental. Except for  $7.4 \mu\text{M}$  La, which reduced RER by 40%, the higher level of each trace metal decreased RER to  $21\pm 9\%$  of the control (c.f. an intended value of 15%).

In Experiment 2, there was good growth of cowpea seedling roots in the control solutions (Fig. 3). For the first 60 h, a RER= $1.54\pm 0.18$  mm/h ( $n=28$ ) was recorded, slightly less than in Experiment 1 in which roots were also grown for 60 h after transplanting. Thereafter, there was a slowing of root growth in the



**Fig. 1** Root elongation rate of cowpea seedlings in the control solution (960  $\mu\text{M}$  Ca, 5  $\mu\text{M}$  B, pH 5.4) (■), during a 4 h exposure period (shaded area) to measured concentrations of Al, Cu, Ga, Gd, Hg, In, La, Ru, or Sc, and during a 48 h recovery period in 960  $\mu\text{M}$  Ca and 5  $\mu\text{M}$  B solutions (pH 5.3).

Relative root elongation rate was calculated for each time interval and plotted against the mid-point of that interval. Vertical bars indicate the standard error of the mean of two replications

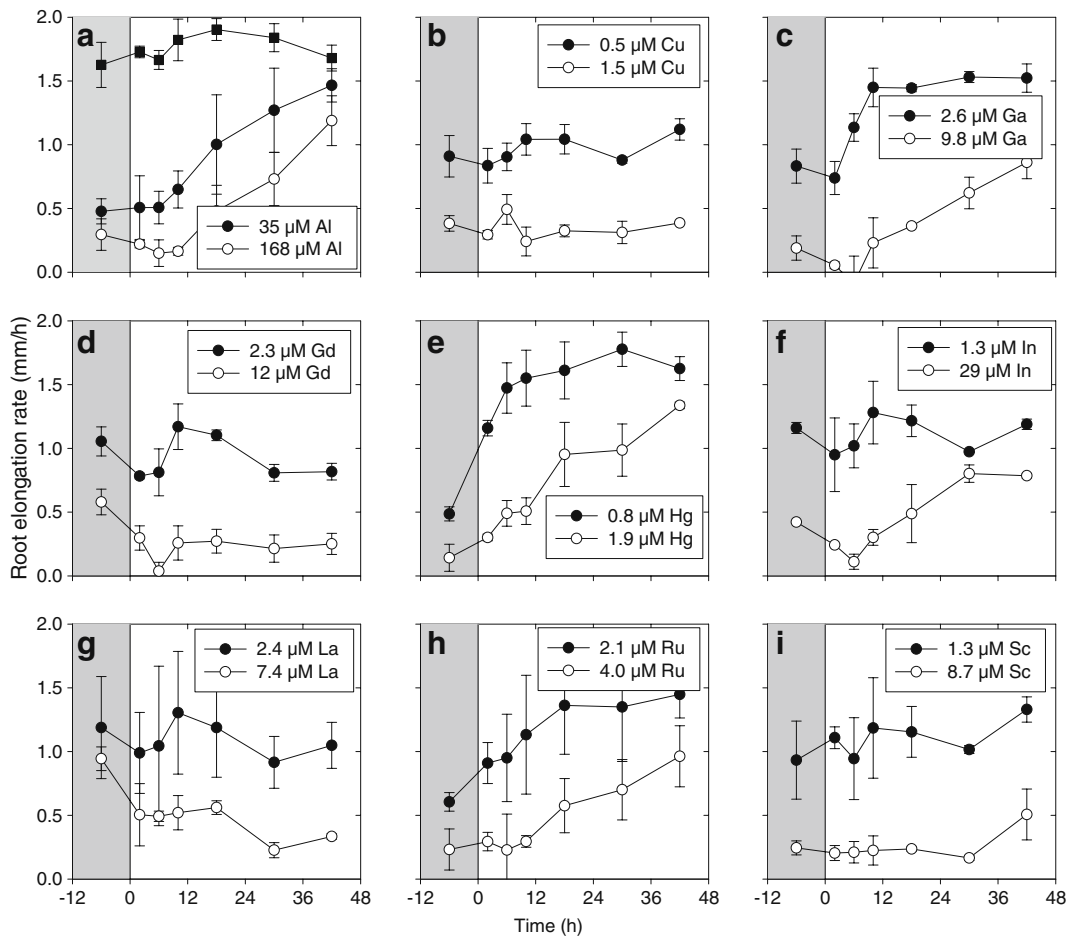
control treatment ( $\text{RER} = 0.97 \pm 0.24$  mm/h,  $n = 6$ ), possibly due to either a decrease in seed reserves or deficiencies in essential nutrients other than Ca and B.

Exposure to trace metals for 12 h produced results similar to those in Experiment 1, with the mean RER of the four trace metals being 18% of the control (Fig. 3a). An increase in the time of exposure to the trace metals had similar effects, except for the 36 h period during which there was a less detrimental effect of Al or Ga than that of Hg or Ru. On average over all exposure periods, Al reduced root growth to 16% of that of roots in the control treatment; corresponding values for Ga, Hg, and Ru were 12, 15 and 17% (Fig. 3).

### Root growth recovery

Roots recovered satisfactorily from exposure for 4 or 12 h to Al, Ga, Hg, and Ru (Fig. 1). Rather surprisingly, given its toxicity, there was rapid recovery in the growth of roots exposed for 4 or 12 h to 0.8 and 1.9  $\mu\text{M}$  Hg. With Al, Ga, or Ru, however, there was a delay of  $\geq 12$  h before growth improved, often preceded by a slight decrease in RER. There was almost complete recovery from 4 h exposure to the lower concentrations of each of these four trace metals (Fig. 1). The higher concentration had a more severe effect, except in the case of Al and Hg.





**Fig. 2** Root elongation rate of cowpea seedlings in the control solution (960  $\mu\text{M}$  Ca, 5  $\mu\text{M}$  B, pH 5.4) (■), during a 12 h exposure period (shaded area) to measured concentrations of Al, Cu, Ga, Gd, Hg, In, La, Ru, or Sc, and during a 48 h recovery period in 960  $\mu\text{M}$  Ca and 5  $\mu\text{M}$  B solutions (pH 5.3).

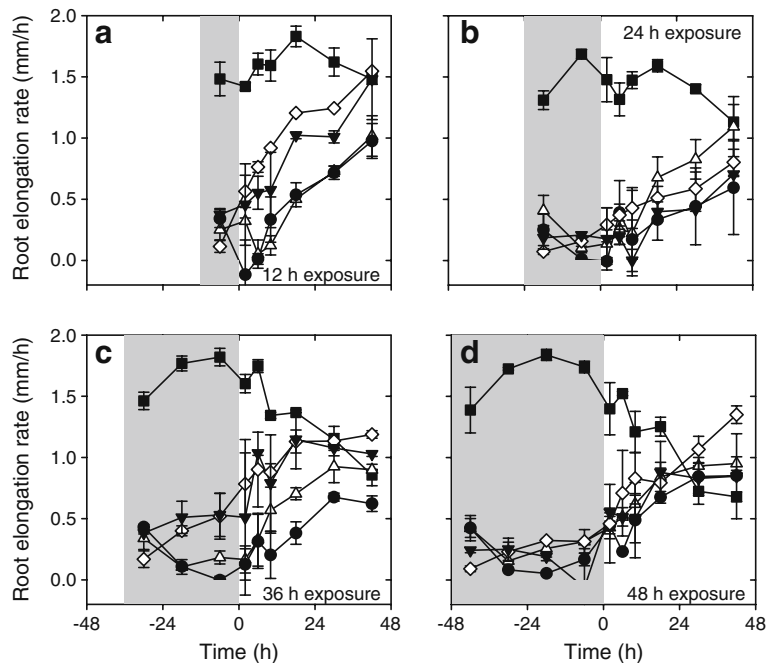
In contrast to the effects of Al, Ga, Hg, or Ru, there was only limited regrowth following exposure of roots to Cu, Gd, In, La, or Sc. There appeared to be an initial recovery in root growth from 4 h exposure to 0.6  $\mu\text{M}$  Cu but stabilised at 85% of the control; stabilisation was at 75% with 1.6  $\mu\text{M}$  Cu. Exposing roots to Cu for 12 h was even more detrimental, with poor growth over the 48 h recovery period. There was a further reduction in RER after exposure for 4 h to Gd and La, followed by some recovery that was 25–50% of the control value. These adverse effects were more evident after exposure to Gd and La for 12 h. Roots grown for 4 or 12 h in solution with 1.3  $\mu\text{M}$  In had poor subsequent growth. It was rather surprising

Relative root elongation rate was calculated for each time interval and plotted against the mid-point of that interval. Vertical bars indicate the standard error of the mean of two replications

that RER increased after 12 h exposure to 29  $\mu\text{M}$  In. In a somewhat similar manner, roots exposed for 4 h to 1.2  $\mu\text{M}$  Sc did not recover well, but better recovery was evident at the higher concentration of 8.4  $\mu\text{M}$  Sc. With 12 h exposure to 1.3 and 8.7  $\mu\text{M}$  Sc, there appeared to be an extended delay (> 36 h) before some recovery was evident. Recovery in RER was < 50% in roots that had been exposed to In or Sc.

The results of Experiment 2 confirmed the recovery of roots from 12 h exposure to the higher concentrations of Al, Ga, Hg, or Ru (Fig. 3a). As in Experiment 1, there was rapid recovery of roots from exposure to Hg, with delays in recovery from the other trace metals. Furthermore, recovery was greatest

**Fig. 3** Root elongation rate of cowpea seedlings in control solution (940  $\mu\text{M}$  Ca, 5  $\mu\text{M}$  B, pH 5.1) (■) during exposure periods of 12–48 h (shaded area) to concentrations of 170  $\mu\text{M}$  Al ( $\Delta$ ), 5.1  $\mu\text{M}$  Ga ( $\bullet$ ), 2.0  $\mu\text{M}$  Hg ( $\diamond$ ), 1.4  $\mu\text{M}$  Ru ( $\blacktriangledown$ ), and during a 48 h recovery period in 940  $\mu\text{M}$  Ca and 5  $\mu\text{M}$  B solutions (pH 5.1). Root elongation rate was calculated for each time interval and plotted against the mid-point of that interval. Vertical bars indicate the standard error of the mean of two replications



from exposure to Hg and least from Ga; recovery from Ru-stress was greater than that from Al-stress. Roots exposed to each of the trace metals for 24–48 h also recovered, with root growth generally increasing after a delay of ca. 12 h except in the case of Hg; roots recovered rapidly in this instance. Over the last 12 h of the experiment, roots that had been exposed to Al for 12 to 48 h had an average RER =  $0.99 \pm 0.08$  mm/h. Corresponding values for Hg- and Ru-stressed roots were  $1.22 \pm 0.32$  and  $1.02 \pm 0.34$  mm/h. Poorest recovery was with Ga-stressed roots, but even these grew at  $0.76 \pm 0.18$  mm/h. Direct comparison with the growth of control roots is difficult, however, given their decline in RER from ca. 60 h after transplanting (Fig. 3c, d).

### Symptoms

In Experiment 1, there were marked visible differences among the trace metals in the symptoms that developed on roots during the recovery period. By the end of the 48 h recovery period, lateral roots developed close to the root tip when exposed for 12 h to 1.8 or 3.8  $\mu\text{M}$  Ru and to the higher rate of Cu, Ga, In, La, or Sc; this was not as evident with Al or Gd. Only a few lateral roots developed after exposure to either 0.8 or 1.9  $\mu\text{M}$  Hg; the roots, though shorter, appeared similar to those in the control treatments. In Experiment 2, lateral root

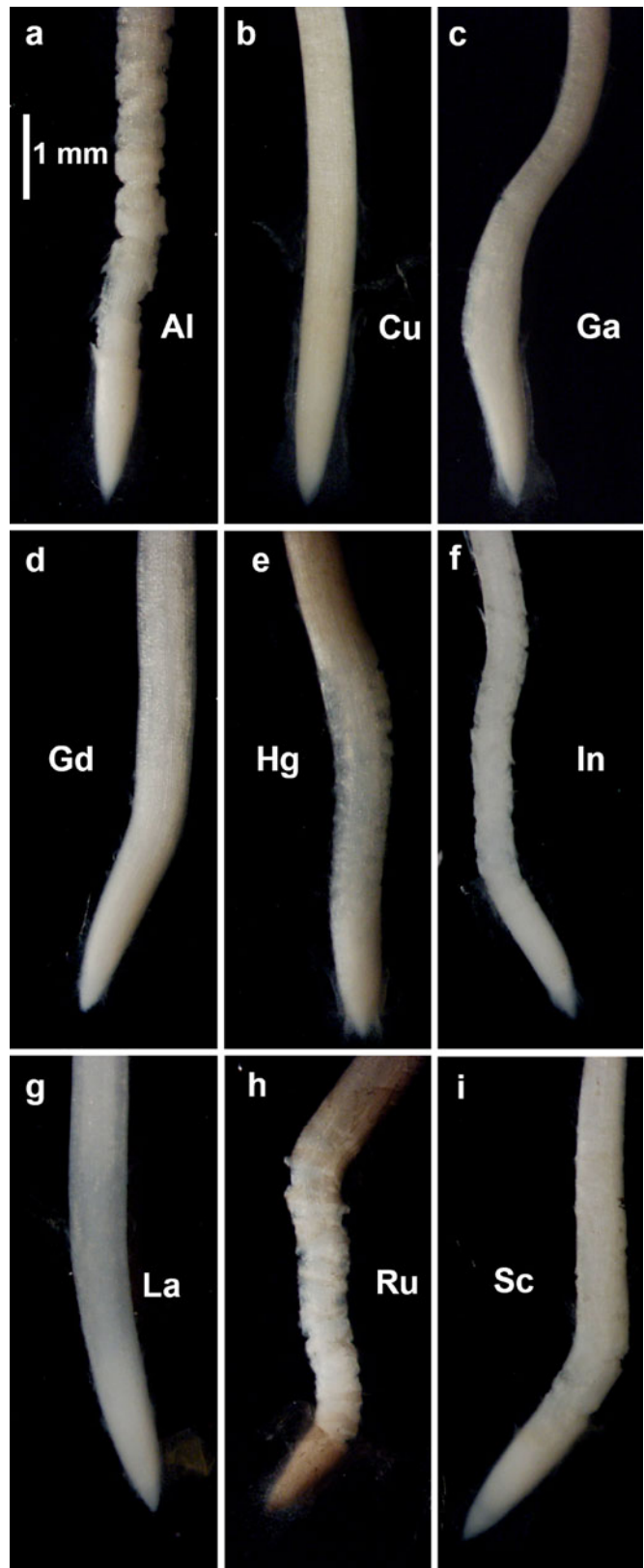
development after 12 h exposure was similar to that in Experiment 1. With extended exposure (especially for 36 and 48 h), however, there was prolific lateral root development in Al-, Ga-, and Ru-stressed roots. While some lateral roots developed after exposure to Hg, primary root regrowth was the more evident.

It was at the microscopic level that trace metals caused clear differences in the primary roots during the recovery period in Experiment 1. Symptoms included ruptures to the rhizodermis and outer cortex similar to those evident during trace metal exposure (see Kopittke et al. (2008) and Kopittke et al. (2009) for light and scanning electron micrographs). These were more severe in roots exposed for 12 h to the higher level of each trace metal (these symptoms being described hereafter). At the time of transfer to the recovery solutions, roots exposed to Al or Ru were severely ruptured and kinked close to the root tip. Mild ruptures and kinks developed in In- and Sc-stressed roots, while kinks but no ruptures developed in roots that had been exposed to Ga and Gd. No symptoms were evident in roots that had been exposed to Cu, Hg, or La.

After 8 h of recovery, the appearance of Al-stressed roots could not be distinguished from those at the time of transfer (Fig. 4a). Roots that had been exposed to Cu or La appeared normal (Fig. 4b, g), as did those exposed to Gd except for a kink ca. 2 mm



**Fig. 4** Light micrographs of unstained cowpea (cv. Caloona) seedling roots after 8 h in recovery solutions (960  $\mu\text{M}$  Ca, 5  $\mu\text{M}$  B, pH 5.3) after growing for the previous 12 h in solutions of 960  $\mu\text{M}$  Ca and 5  $\mu\text{M}$  B plus ( $\mu\text{M}$ ) **a** 170 Al, **b** 1.5 Cu, **c** 7.1 Ga, **d** 12 Gd, **e** 1.9 Hg, **f** 29 In, **g** 7.4 La, **h** 4.0 Ru, or **i** 8.7 Sc. The 1 mm scale bar in (a) applies to all images



behind the tip (Fig. 4d). Roots that had been exposed to Ga were swollen ca. 1 mm behind the tip and mild rupturing had developed (Fig. 4c). Ruptures developed in roots that had been exposed to Hg (Fig. 4e), despite good root regrowth of Hg-intoxicated roots (Fig. 2e). Rupturing was more severe in the case of In or Sc (Fig. 4f, i).

After 24 h of recovery, primary root growth of Al-stressed roots was normal (smooth rhizodermis and typical root cap) except for the ruptures that had developed during the period in Al solution (Fig. 5a). A few lateral roots emerged near the ruptured region 36 h from the start of the recovery period; this was clearly evident 12 h later (Fig. 5f). Roots that had been exposed to Cu showed no morphological damage except for a light brown discolouration 1–2 mm behind the root tip. A clear change in root direction accompanied by swelling was evident in roots that had been exposed to Ga or Gd. Lateral roots had emerged from the swollen portion of the root 48 h after transfer (Fig. 5g, h). Normal primary root growth of Hg-stressed roots had commenced by 24 h after transfer, though the region exposed to Hg was discoloured (light brown) along with a short section of ruptures (Fig. 5b). A protuberance (bump) developed around the root at the site of the ruptures, from which region a few lateral roots had emerged 48 h after transfer (Fig. 5i). There appeared to be some regrowth of the primary root of In-intoxicated roots, though the root cap appeared to be slightly detached from the root tip and the rhizodermis was not smooth (Fig. 5c); these symptoms were still evident 24 h later. No symptoms were evident in roots exposed to La for up to 48 h after transfer. Normal primary root growth was evident 24 h after transfer from solutions containing Ru despite the severe rupturing and deformities that had previously developed (Fig. 5d). Lateral roots later developed from the ruptured region (Fig. 5j). The mild ruptures present at the time of transfer of Sc-stressed roots had become more severe 24 h later, with the root tip becoming swollen and deformed (Fig. 5e). This was even more evident 24 h later, with many lateral roots emerging from the swollen tip (Fig. 5k). In many instances, the root emerging from the apex had extended by 1–2 mm, probably accounting for the observed late regrowth of Sc-stressed roots (Fig. 2i). It is not known, however, if this constitutes growth of the primary meristem or of a lateral root.

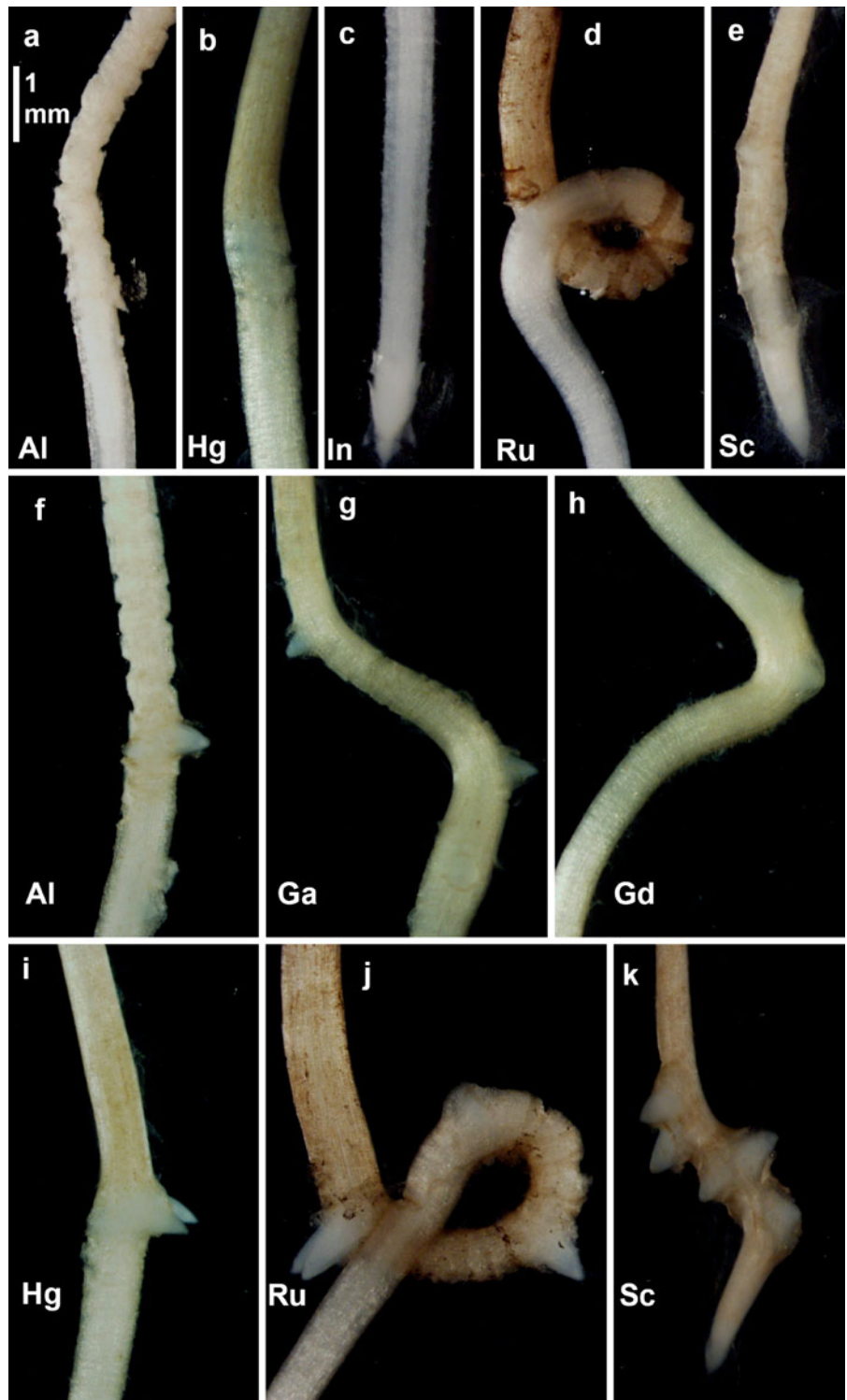
It is noteworthy that exposure to trace metals had no observable detrimental effects on shoot growth during the exposure or recovery periods, despite the varied adverse effects on root growth. Hypocotyl and epicotyl elongation, the appearance of the cotyledons, the expansion of the first true leaves, and the appearance of the apical meristem were all normal.

## Discussion

It is becoming increasingly clear that biological effects should be related to the measured concentrations of trace metals because of the considerable loss of some trace metals from solution (Lee et al. 2005). Reid et al. (1996), in research on *Chara corallina* (Klien ex. Willd., em. R.D.W.), noted “the rapid depletion of trivalent cations from solution by adsorption to cell walls” with the concentrations of Ga and Sc often falling by >50% within 0.5 h. In the present study (Tables 1 and 2) and in that of (Kopittke et al. 2009), there were marked reductions in Ga, In, Ru, and Sc in solution even before transfer of seedlings, indicating loss through precipitation or adsorption by the glass beaker. There was further loss by the end of the experimental period (Tables 1 and 2), making it difficult to relate toxicity to metal concentration in solution (for this study, growth was related to the mean of the values measured at the beginning and end of the exposure period). Losses from solution indicate that some trace metals are more highly toxic than initially thought based on results using nominal concentrations. Although solutions containing Al, Cu, Gd, or La were dominated by the free ion, other solutions were dominated by Ga  $(\text{OH})_2^+$ , HgCl<sub>2</sub><sup>0</sup>, In(OH)<sub>2</sub><sup>+</sup>, or ScOH<sup>2+</sup> (no data were available for Ru). These differences in speciation hinder efforts to determine the rhizotoxic species (see Kinraide (1991) for a discussion of Al), and for most of these other metals, the low pH required to ensure that the free ion dominated would completely inhibit root growth.

The results of Experiment 1 (Figs. 1 and 2) demonstrate clear differences in the ability of primary roots of cowpea (cv. Caloona) seedlings to recover over a 48 h period after exposure to concentrations of trace metals that decrease RER by 50 or 85%. Root growth recovered after 4 or 12 h exposure in solutions containing Al, Ga, Hg, and Ru. In contrast, there was

**Fig. 5** Light micrographs of unstained cowpea (cv. Caloona) seedling roots after 24 (a–e) or 48 h (f–k) in recovery solutions (960  $\mu\text{M}$  Ca, 5  $\mu\text{M}$  B, pH 5.3) after growing for the previous 12 h in solutions of 960  $\mu\text{M}$  Ca and 5  $\mu\text{M}$  B plus ( $\mu\text{M}$ ) a, f 170 Al, b, i 1.9 Hg, c 29 In, d, j 4.0 Ru, e, k 8.7 Sc, g 7.1 Ga, or h 12.2 Gd. The 1 mm scale bar in (a) applies to all images



no recovery (or recovery that was only limited and delayed) after roots had been grown in solutions of Cu, Gd, In, La, and Sc.

Recovery of root growth after exposure for up to 12 h to  $\leq 170$   $\mu\text{M}$  Al supports the conclusion of Bennet and Breen (1991) that “resumption of primary growth in Al-damaged roots ... indicates that the mitotically active cells of the cap and root are not permanently affected by Al”. This has been confirmed by recent evidence using Evans blue staining, cell death occurring in the Al-induced ruptured region but not at the root apex (Motoda et al. 2010). Al-induced damage of cells in the root tip of an Al-sensitive maize line was also evident in the study of Amenós et al. (2009) but root growth recovered to control values after 6 h in Al-free solution. Findings of Doncheva et al. (2005) are noteworthy in that cell division was inhibited in the proximal meristem (0.25–0.80 mm) of Al-sensitive maize roots 5 min after exposure to 50  $\mu\text{M}$  Al. After 10–30 min, cell division occurred in the distal elongation zone (2.5–3.1 mm), followed by the development of an incipient lateral root after 180 min.

As with Al, recovery from exposure to 2.2 and 8.5  $\mu\text{M}$  Ga, 0.8 and 1.9  $\mu\text{M}$  Hg, or 1.8 and 3.8  $\mu\text{M}$  Ru (mean measured values) suggests that these four trace metals do not permanently damage meristematic root cells of cowpea at these concentrations. It would appear, in contrast, that meristematic cells of roots are damaged when grown for 4 or 12 h in solutions that contain Cu, Gd, In, La, and Sc at concentrations that decrease root growth by up to 85%. Patra et al. (2004) classified the effects of some trace metals on cell division, with Al having a less detrimental effect than did Cu; in the present study, roots recovered from Al-stress but not from Cu-stress. However, Patra et al. (2004) found that both Cu and Hg had marked detrimental effects on mitosis; roots recovered from Hg intoxication but not from exposure to Cu in the present study.

Many studies have used Ga, Gd, La, and Sc to gain insight into the effects of soluble Al (Bennet and Breen 1991; Clarkson 1965; Hou et al. 2010; Reid et al. 1996), a major problem in acid soils (von Uexküll and Mutert 1995). The results of the present study and those of Kopittke et al. (2008) and Kopittke et al. (2009) have demonstrated some limitations in this approach. Differences are evident among trace metals

in the concentrations which are toxic, symptoms caused, the kinetics of toxic effects, and recovery from intoxication. The toxic effects of Ga differ from those of Al in the range in concentration that cause ruptures, the severity of the ruptures, but roots recover from exposure to Ga as they do from Al intoxication (Figs. 1 and 2). In contrast, there was no recovery from exposure to Gd or La, and there was a narrow range in concentrations of these metals that caused ruptures. Hou et al. (2010) concluded that  $\text{Al}^{3+}$  and  $\text{La}^{3+}$  behave similarly at the cell surface, but found that La is more toxic than Al and that La did not increase abscisic acid in soybean shoots though both trace metals did so in the roots. As found by Reid et al. (1996) and in the present study, Sc is considerably more toxic than Al. Caution is advised in the use of La as a Ca-channel blocker in root cell membranes because of the complexity of its toxic effects, including rupturing of cell walls.

The development of lateral roots, indicating a loss of apical dominance, during the recovery period suggests that cells of the pericycle, from which lateral roots develop, are not damaged by exposure to toxic concentrations of the trace metals tested. Indeed, lateral roots developed in the ruptured region in some instances. Furthermore, the study by Doncheva et al. (2005) demonstrates that cell division occurs in the distal elongation zone of Al-sensitive maize roots 10–30 min after exposure to Al, with evidence of lateral root development in this zone after 180 min.

While there are benefits in the techniques used in the present study, it is important to note that these toxic effects of trace metals apply to short-term root growth of cowpea (cv. Caloona) seedlings in simplified nutrient solution. Specifically, the results apply to growth in which seed nutrient reserves are utilized and not to conditions in which plants are dependent for growth on photosynthesis or on the uptake of nutrients other than Ca and B.

## Conclusions

Results of the present study have confirmed differences in the toxicity of Al, Cu, Ga, Gd, Hg, In, La, Ru, or Sc (metals that cause rupturing to the outer layers of roots) in measured concentrations that decrease cowpea root growth (Kopittke et al. 2008; Kopittke et al. 2009). Further, there were marked

differences in recovery from exposure to these trace metals at concentrations that cause a 50 or 85% reduction in RER. Specifically, roots recovered rapidly and completely from exposure to Hg, despite its highly toxic effects. Recovery was delayed or somewhat limited for the next 48 h after exposure to Al, Ga, or Ru, in keeping with results of Bennet et al. (1991), Wagatsuma et al. (2005), and Motoda et al. (2010) regarding Al. Recovery of cowpea roots from exposure to these metals suggests continued function of meristematic root cells despite evidence of cell damage by Al (Amenós et al. 2009; Doncheva et al. 2005). This contrasted to Cu, Gd, In, La, or Sc from which there was no recovery or only minor recovery. While some common mechanisms of rhizotoxicity exist, the differences in concentrations that decrease root growth, the rapidity with which growth is reduced, the symptoms that develop (either during exposure or during the recovery period), and the ability to recover (kinetics or magnitude) suggest that each trace metal has a distinctive combination of rhizotoxic effects. These differences may help determine the underlying causes of rhizotoxicity, but counsel caution in the use of trace metals as analogues for studying the physiological effects of Al.

**Acknowledgments** The authors thank Jason Phipps for providing initial information on the toxic effects of trace metals. This research was funded through the Cooperative Research Centre for Contamination Assessment and Remediation of the Environment (CRC-CARE) Project 3-03-05-09/10, through the Australian Research Council's (ARC) Discovery funding scheme (DP0665467), and The University of Queensland Early Career Researcher scheme (2008003392).

## References

- Amenós M, Corrales I, Poschenrieder C, Illes P, Baluska F, Barceló J (2009) Different effects of aluminum on the actin cytoskeleton and brefeldin A - sensitive vesicle recycling in root apex cells of two maize varieties differing in root elongation rate and aluminum tolerance. *Plant Cell Physiol* 50:528–540
- Babula P, Adam V, Opatrilova R, Zehnalek J, Havel L, Kizek R (2008) Uncommon heavy metals, metalloids and their plant toxicity: A review. *Environ Chem Lett* 6:189–213
- Bennet RJ, Breen CM (1991) The recovery of roots of *Zea mays* L. from various aluminium treatments: Towards elucidating the regulatory processes that underlie root growth control. *Environ Exp Bot* 31:153–163
- Blamey FPC, Nishizawa NK, Yoshimura E (2004) Timing, magnitude, and location of initial soluble aluminium injuries to mungbean roots. *Soil Sci Plant Nutr* 50:67–76
- Buckingham S, Maheswaran J, Meehan B, Peverill K (1999) The role of application of rare earth elements in enhancement of crop and pasture production. *Mater Sci Forum* 315:339–347
- Clarkson DT (1965) The effect of aluminium and some other trivalent metal cations on cell division in root apices of *Allium cepa*. *Ann Bot* 29:309–315
- Doncheva S, Amenós M, Poschenrieder C, Barceló J (2005) Root cell patterning: a primary target for aluminium toxicity in maize. *J Exp Bot* 56:1213–1220
- Hou N, You J, Pang J, Xu M, Chen G, Yang Z (2010) The accumulation and transport of abscisic acid in soybean (*Glycine max* L.) under aluminum stress. *Plant Soil* 330:127–137
- Kinraide TB (1991) Identity of the rhizotoxic aluminium species. In: Wright RJ, Baligar VC, Murrmann RP (eds) *Plant-Soil Interactions at Low pH*. Developments in Plant and Soil Sciences 45. Kluwer Acad. Publ, Dordrecht, pp 717–728
- Kollmeier M, Felle HH, Horst WJ (2000) Genotypical differences in aluminum resistance of maize are expressed in the distal part of the transition zone. Is reduced basipetal auxin flow involved in inhibition of root elongation by aluminum? *Plant Physiol* 122:945–956
- Kopittke PM, Blamey FPC, Menzies NW (2008) Toxicities of soluble Al, Cu, and La include ruptures to rhizodermal and root cortical cells of cowpea. *Plant Soil* 303:217–227
- Kopittke PM, McKenna BA, Blamey FPC, Wehr JB, Menzies NW (2009) Metal-induced cell rupture in elongating roots is associated with metal ion binding strengths. *Plant Soil* 322:303–315
- Lee DY, Fortin C, Campbell PGC (2005) Contrasting effects of chloride on the toxicity of silver to two green algae, *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii*. *Aquat Toxicol* 75:127–135
- McLaughlin MJ, Tiller KG, Naidu R, Stevens DP (1996) Review: The behaviour and environmental impact of contaminants in fertilizers. *Aust J Soil Res* 34:1–54
- Motoda H, Kano Y, Hiragami F, Kawamura K, Matsumoto H (2010) Morphological changes in the apex of pea roots during and after recovery from aluminium treatment. *Plant Soil* 333:49–58
- Parkhurst D 2007 PhreeqcI v2.13.04. United States Geological Survey. <http://water.usgs.gov/owq/software.html>.
- Patra M, Bhowmik N, Bandopadhyay B, Sharma A (2004) Comparison of mercury, lead and arsenic with respect to genotoxic effects on plant systems and the development of genetic tolerance. *Environ Exp Bot* 52:199–223
- Rangel AF, Rao IM, Horst WJ (2007) Spatial aluminium sensitivity of root apices of two common bean (*Phaseolus vulgaris* L.) genotypes with contrasting aluminium resistance. *J Exp Bot* 58:3895–3904
- Reid RJ, Rengel Z, Smith FA (1996) Membrane fluxes and comparative toxicities of aluminium, scandium and gallium. *J Exp Bot* 47:1881–1888
- Scott BJ, Ewing MA, Williams R, Humphries AW, Coombes NE (2008) Tolerance of aluminium toxicity in annual *Medicago* species and lucerne. *Aust J Exp Agric* 48:499–511
- Sivaguru M, Horst WJ, Eticha D, Matsumoto H (2006) Aluminum inhibits apoplastic flow of high-molecular

- weight solutes in root apices of *Zea mays* L. J Plant Nutr Soil Sc 169:679–690
- Taylor GJ, Stadt KJ, Dale MR (1991) Modelling the phytotoxicity of aluminum, cadmium, copper, manganese, nickel, and zinc using the Weibull frequency distribution. Can J Bot 69:359–367
- von Uexküll HR, Mutert E (1995) Global extent, development and economic impact of acid soils. Plant Soil 171:1–15
- Wagatsuma T, Ishikawa S, Uemura M, Mitsuhashi W, Kawamura T, Khan MSH, Tawarayama K (2005) Plasma membrane lipids are the powerful components for early stage aluminum tolerance in triticale. Soil Sci Plant Nutr 51:701–704