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Toxic effects of low concentrations of Cu on nodulation of cowpea (*Vigna unguiculata*)

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Abstract

Although Cu is phytotoxic at Cu²⁺ activities as low as 1-2 µM, the effect of Cu²⁺ on the nodulation of legumes has received little attention. The effect of Cu²⁺ on nodulation of cowpea (*Vigna unguiculata* (L.) Walp. cv. Caloona) was examined in a dilute solution culture system utilizing a cation exchange resin to buffer solution Cu²⁺. The nodulation process was more sensitive to increasing Cu²⁺ activities than both shoot and root growth; whilst a Cu²⁺ activity of 1.0 µM corresponded to a 10 % reduction in the relative yield of the shoots and roots, a Cu²⁺ activity of 0.2 µM corresponded to a 10 % reduction in nodulation. This reduction in nodulation with increasing Cu²⁺ activity was associated with an inhibition of root hair formation in treatments containing ≥ 0.77 µM Cu²⁺, rather than to a reduction in the size of the *Rhizobium* population.

Capsule: The nodulation process was more sensitive to increasing Cu²⁺ activities than either shoot or root growth.

Keywords: Copper (Cu), legumes, nodulation, phytotoxicity symptoms, *Rhizobium*, root hairs

Introduction

Although toxic Cu concentrations have been observed on naturally 'polluted' soils (for example, Cu ore outcrops) (Eleftheriou and Karataglis, 1989), Cu toxicity is most often of concern on anthropogenically polluted soils (such as from mining, smelting, waste disposal, and the use of fertilizers and fungicides). When present at elevated concentrations, Cu is highly toxic to plant growth (Taylor and Foy, 1985; Zhu and Alva, 1993). Indeed, Cu²⁺ is as toxic (if not more) than the various monomeric Al species; Cu²⁺ activities of less than 1 or 2 µM significantly reduced the shoot and root mass of Rhodes grass (*Chloris gayana*) (Sheldon and Menzies, 2005) and cowpea (*Vigna unguiculata*) (Kopittke and Menzies, 2006).

In addition, anthropogenically polluted soils (particularly those from mining) often contain low levels of organic matter, and hence growth on such soils is often limited by N deficiency. Thus, revegetation strategies often utilize leguminous species to improve the soil's N-status. However, Cu reduced and inhibited root hair growth of Rhodes grass and cowpea at concentrations smaller than that needed to cause a significant reduction in root mass (Kopittke and Menzies, 2006; Sheldon and Menzies, 2005). With Al, a reduction in root hair growth (at concentrations smaller than that required to reduce root growth *per se*) was associated with a decrease in the nodulation of soybean (*Glycine max*) and two *Stylosanthes* spp. (Alva et al., 1987; de Carvalho et al., 1982). Large Cu concentrations may similarly inhibit the nodulation of leguminous species. Hallsworth et al. (1964) showed that Cu added in solution to a sandy soil at 10 µM inhibited nodule formation and development of subterranean clover (*Trifolium subterraneum*).

This study examines the effects of increasing Cu concentrations on the survival of *Rhizobium* and on the root hair formation and nodulation of cowpea (*Vigna unguiculata*). Optical microscopy examined changes in root structure with increasing Cu²⁺ activity.

Materials and Methods

Resin preparation and solution culture

The Cu in the nutrient solutions was buffered through the use of a cation exchange resin. The resin and the solution culture system were prepared as described by Kopittke & Menzies (2006). Approximately 400 g of Amberlite IRC-748 (Aldrich) ion exchange resin was converted to either the Cu- or Ca-form at pH 4.50. The Cu and Ca resins were mixed at eight ratios (1.3, 5.0, 20, 45, 62.5, 67.5, 75, and 85 % Cu-form resin) so as to yield a total resin mass of 10.5 g (i.e. 0.5 g resin/L of the final nutrient solution).

Following mixing, 20 mL of 2 mM CaCl₂ was added to each resin mixture and the resins allowed to equilibrate for 14 d. In addition, 0.1 g of Mn-saturated resin (oven-dry equivalent) and 0.2 g of Zn-saturated resin (oven-dry equivalent) was added to each container in order to supply sufficient Mn and Zn (preliminary experiments indicated that the Cu/Ca resin removed both Mn and Zn from the nutrient solution resulting in nutritional deficiencies).

Experiments were carried out in controlled glasshouse conditions, with three high pressure sodium lamps providing 16 h of light per day (photosynthetically active

radiation of 400 $\mu\text{mol}/\text{m}^2/\text{s}$ at plant height). Temperature was maintained at 27 °C during the light period and 25 °C during the dark.

Twenty four polypropylene containers (22 L; 265 mm diameter by 400 mm deep) were arranged in a completely randomized design with a total of eight treatments (1.3, 5.0, 20, 45, 62.5, 67.5, 75, and 85 % Cu-form resin) and three replicates. The resin was held in a PVC container with 150 μm mesh ends. An air-lift water pump was used to force the solution through the resin, and also to aerate the solution. After an initial 24 h aeration, each container was connected to a separate pH titration unit (TPS, miniCHEM-pH) and peristaltic pump (Masterflex 5 RPM with Masterflex Tygon tubing, L/S 17) which was used to raise and maintain the pH at 4.5 by the addition of 0.025 M $\text{Ca}(\text{OH})_2$. The Cu/Ca resin mixtures were added to each pot, 0.5 g of the Zn resin and 0.25 g of the Mn resin incorporated, and the solutions allowed to equilibrate for 21 d. After equilibration, the nutrient solution was found to contain 0.2-2.7 μM Cu depending upon treatment, and 0.5-1.0 μM Zn, and 0.3-0.6 μM Mn.

Nutrients were supplied through the use of a dilute basal nutrient solution (added upon initiation of the experiment), and the incremental addition of a delivery nutrient solution at quantities calculated to meet plant demand. Each container was filled with a basal nutrient solution, with the Cu supplied from the ion exchange resins (Table 1). To minimize Cu complexation, Fe was supplied as FeCDTA (see Kopittke & Menzies (2006)). No N was added (either in the basal or delivery solutions) so as to encourage nodulation. In addition to this basal nutrient solution, nutrients were supplied to the

plants through the addition of a pre-calculated quantity of delivery nutrient solution (see Kopittke & Menzies (2006)) (Table 1).

Rhizobium inoculation and population

Cowpea seeds (*Vigna unguiculata* (L.) Walp. cv. Caloona) were imbibed in aerated 200 μM CaSO_4 solution for 2 h following seed surface sterilisation (40 mM sodium hypochlorite (NaOCl) for 4 min and deionised (DI) water (changed regularly) for 2 h). Seeds were rolled in paper towel, and germinated for 36 h at 30 °C with the ends of the paper towel immersed in tap water. Five seedlings with radicle lengths of 10 ± 2 mm were transferred to each 22 L container for 48 h (the beginning of this 48 h growth period being 0 days after planting (DAP)). The zone of emerging root hairs is known to be the infective region of the root (Calvert et al., 1984), thus, to minimize nodulation from pre-existing root hairs, plants were grown at the appropriate Cu level for 48 h prior to inoculation. After this 48 h growth period, a dense suspension (approximately 10^8 cells/mL) of the cowpea-type *Bradyrhizobium* strain CB756 was prepared. A series of flat, 1 L containers were filled with sterilized DI water and 10 mL of the *Rhizobium* suspension added to each container to give an inoculum of approximately 10^6 cells/mL. So as to ensure a high *Rhizobium* population in the rhizosphere, the seedlings were removed from the 22 L containers and the radicles carefully placed in these 1 L containers for 2 h (this is 0 days after inoculation (DAI), i.e. 2 DAP). At the same time, 15 mL of the *Rhizobium* suspension was placed into each of the 22 L containers and allowed to mix for 2 h. This was calculated to develop an initial population of approximately 10^5 cells/mL. After this 2 h period, the seedlings were removed from the

1 L containers and placed back into the appropriate 22 L containers. The aeration stream was disconnected for 10 h so as to enable the *Rhizobium* to establish in the rhizosphere.

A plant infection dilution count in tube culture was used to estimate the number of rhizobia in the solution culture system during the experimental period. The basic procedure is described by Brockwell (1980) with the following modifications. Siratro seeds (*Macroptilium atropurpureum* (DC) Urban (atro) cv. Aztec) were placed in a desiccator for 2 d, soaked in dry, concentrated H₂SO₄ for 10 min, rinsed nine times with sterilized DI water, and then soaked in sterilized DI water for 6 h. The siratro seeds were then removed and allowed to germinate overnight at 28 °C on a sterilized water agar (1.5 %). Nitrogen-free seedling agar was then prepared as described by Jensen (1942) with 1 mL/L of trace element solution added (Gibson, 1963), and 15 mL sloped in a series of glass tubes (150 x 25 mm) closed with cotton wool. The siratro seeds (with radicles 5-10 mm in length) were transferred from the water agar to the nitrogen-free seedling agar in the glass tubes (one per tube), and allowed to grow for 2 d. A total of 864 glass tubes/seedlings were prepared.

Of the eight treatments and three replicates comprising this experiment, four of the treatments (1.3, 45, 67.5, and 85 % Cu resin) and two of the replicates were selected for the plant infection count. The *Rhizobium* population was assessed in the bulk nutrient solution of these selected treatments at four time intervals (0, 3, 8, and 12 DAI), and in the rhizosphere at two time intervals (3 and 12 DAI). A ten-fold dilution series (1 + 9 mL) was utilized for all samples to 10⁻⁶, thus yielding a total of 864 samples. For each sample, 1 mL was pipetted into each of three replicate plant tubes, ensuring good

contact between the solution and the siratro radicle. The siratro test plants were allowed to grow in a temperature controlled glasshouse for three weeks after inoculation before being examined for the presence of nodules. The most probable number of nodule bacteria present in each of the samples was then calculated based upon the distribution of positive (nodulated) siratro plants in each level of the dilution series (see Brockwell (1980)). The rhizosphere *Rhizobium* population was determined by destructively harvesting one root from each of the selected treatments and placing it in a 28 mL McCartney bottle with 10 mL DI water and glass beads. The bottle was shaken rapidly on a wrist-action shaker for 15 min, and the solution analyzed as described above to estimate the number of rhizobia.

Solution and plant analysis

The electrical conductivity (EC) of the nutrient solution was measured twice weekly. Nutrient solution samples were taken on days 0, 5, 10, and 16, filtered (0.22 µm Millipore GSWP), acidified to pH < 2.0 using 20 µL of concentrated HCl, and refrigerated (3.5 °C) before analysis by inductively coupled plasma atomic emission spectrometry (ICPAES) for K, Mg, S, Ca, and Fe, and by inductively coupled plasma mass spectrometry (ICPMS) for Cu, Zn, and Mn. Upon completion of the experiment (16 DAP), selected roots were taken from each treatment, stained with 0.5 % toluidine blue O, and examined under a stereo microscope. The number of nodules per plant, and the nodule location (primary or secondary root) were recorded, and the roots thoroughly rinsed in DI water (5 mins). Plants were dried for 3 d at 65 °C, and dry masses of the roots and shoots determined. Shoot and root N concentrations were measured as detailed by Rayment & Higginson (1992) using a LECO CNS 2000, and all other

elements determined by ICPAES after acid digestion as described by Martinie & Schilt (1976).

Data analysis

For relative nodule number and relative shoot and root mass, a grouped generalised linear regression analysis (logistic link) was performed using GenStat 7 (GenStat, 2003). All data was fitted using Cu concentrations as determined upon completion of the trial (16 DAP), with calculations of ionic strength (I) and ion activities performed with PhreeqcI 2.11 with the Minteq database (Parkhurst, 2005). Chelated Cu accounted for < 1.3 % of the total solution Cu, with Cu^{2+} accounting for > 99.9 % of the remaining soluble Cu forms (see Kopittke & Menzies (2006) for details).

Results

Plant growth, nodulation, and the Rhizobium population

Both the number of nodules per plant and the plant yield were dependent upon the solution Cu^{2+} activity, with the relative number of nodules and the relative root and shoot mass decreasing with increasing Cu^{2+} activity ($F_{5,18} = 60.0$ and $p < 0.001$) (Fig. 1). Closer examination revealed that the root and shoot data could be represented by a single curve, with neither slopes ($F_{1,12} = 1.55$ and $p = 0.237$) nor intercepts ($F_{1,13} = 4.41$ and $p = 0.056$) differing significantly. Reanalysis of the data, grouping roots and shoots together, indicated that the combined root/shoot curve and the nodule curve could be best represented by two separate parallel curves (no significant difference in slopes, $F_{1,20} = 1.84$ and $p = 0.190$, but a significant difference in intercept, $F_{1,20} = 111$ and $p < 0.001$). The mean number of nodules per plant began to decrease at a lower Cu^{2+}

activity than did the relative root/shoot mass (Fig. 1). For the roots and shoots, the critical toxic solution Cu^{2+} activity (corresponding to 90 % maximum growth) was calculated to be 1.0 μM (Fig. 1). In contrast, a Cu^{2+} activity of 0.2 μM was found to correspond to 90 % relative nodulation (Fig. 1).

The first nodules were observed 10 DAI. The overall rate of nodulation was low, even in the lowest Cu treatments where a mean of only three nodules per plant was observed. The number of rhizobia in the bulk nutrient solution (as estimated from the siratro infection test), was variable but generally lower than anticipated, ranging from 10^2 to 10^4 rhizobia per mL at 0 DAI. In addition, the number of rhizobia decreased rapidly with time; this decrease appearing to be largely independent of the nutrient solution Cu^{2+} activity (Fig. 1). Survival of the *Rhizobium* was greater in the rhizosphere than in the bulk nutrient solution at 3 DAI (ranging from 10^3 to 10^4 rhizobia per mL). This decreased to 10^1 to 10^4 by 12 DAI. Rhizosphere populations measured 3 DAI were approximately 10 times greater than those in the bulk solution, while 10 to 1000 times greater 12 DAI (data not presented).

Nutrient solution and plant tissue analysis

Solution Cu^{2+} concentrations remained relatively constant over the 16 d growth period, with concentrations in each treatment generally not varying by more than 15 % (data not presented). Solution concentrations of all other nutrients also tended to remain steady over time, particularly in the low Cu solutions (Fig. 2). Although nutrient additions tended to exceed plant uptake in the higher Cu treatments (where growth rates were lower), increases in concentrations were < 15 % (e.g. Mg in Fig. 2). Changes in solution

EC over time were also small; being balanced by the addition of nutrients and $\text{Ca}(\text{OH})_2$ (as a pH titrant), and the depletion of nutrients by the plant roots (Fig. 2). Solution concentrations of Mn (supplied by the Mn-saturated resin) were higher than had been expected from preliminary experiments, ranging from 0.3 to 0.6 μM . This was most likely due to the displacement of Mn from the Mn-saturated resin by Cu.

Shoot and root Cu concentrations increased with increasing solution Cu^{2+} activity, with Cu concentrations approximately 5-fold greater in the roots than in the shoots (Fig. 3). Although nutrient supply was generally maintained in ranges considered adequate, approximately 4 DAP interveinal chlorosis (similar to that characteristic of Fe deficiency) was observed on the leaves of the plants growing in the two highest Cu solutions. Furthermore, approximately 13 DAP, symptoms of Mn toxicity were observed on the leaves of the plants in all but the two highest Cu^{2+} treatments, with dark brown, necrotic spots forming on the leaf surface. Tissue analysis revealed that shoot Mn concentrations in these lower Cu^{2+} treatments (190-250 $\mu\text{g/g}$) were substantially higher than in the two highest Cu^{2+} treatments (approximately 60 $\mu\text{g/g}$). Concentrations of tissue-N were low (10-17 mg/g for the shoot), although tended to be highest in the two upper Cu^{2+} treatments (Fig. 3). Symptoms of N deficiency (leaves turning a pale green) were particularly prevalent in the low Cu^{2+} solutions. For the remainder of the nutrients in the low Cu^{2+} treatments, shoot concentrations were at levels considered adequate for the growth of cowpea (Reuter and Edwards, 1997) (data not presented). In comparison, shoot concentrations of Ca, Mg, Fe, Zn, and Mn decreased at high Cu^{2+} (data not presented).

Root examination

Root growth during the 16 d experimental period was rapid, with roots in the low Cu solutions reaching lengths of approximately 400 mm. However, roots taken from the two highest Cu treatments, in which growth was significantly reduced (Fig. 1), were dark brown in color. Examination under the optical microscope revealed that, particularly at the highest Cu level, roots had bent tips, displayed an increased number of secondary roots initiated per unit root length, and were short, stubby, and often swollen behind the root tip (Fig. 4). Root hair growth was prolific in the two lowest Cu solutions, with the tips of the root hairs often curled (Fig. 4). However, root hair growth at 0.77 $\mu\text{M Cu}^{2+}$ was greatly reduced, with those hairs that did form often shorter and straighter than those in the two lower Cu treatments (Fig. 4). Other than those most likely present at the time of transfer (i.e. in the top 10-20 mm of the root), very few root hairs were found in roots growing in $\geq 1.0 \mu\text{M Cu}^{2+}$ (Fig. 4).

Discussion

The nodulation process was more sensitive to increasing Cu^{2+} activities than was the growth of the shoots and roots; a Cu^{2+} activity of 1.0 μM causing a 10 % reduction in shoot and root weight, whilst a Cu^{2+} activity of 0.2 μM caused a 10 % reduction in nodulation (Fig. 1). It is considered that nodulation was more sensitive to Cu^{2+} than plant growth *per se* due to the reduction in root hair formation (and hence reduction in potential infection sites) observed in the treatments with Cu^{2+} activities $\geq 0.77 \mu\text{M}$ (c.f. shoot and root weight reduction $\geq 1.0 \mu\text{M Cu}^{2+}$) (Fig. 1 and Fig. 4). Furthermore, although some root hairs were formed in the 0.77 $\mu\text{M Cu}^{2+}$ treatment, these hairs tended to be short and stubby, and were not curled (Fig. 4). Root hair curling precedes infection

by rhizobia (Turgeon and Bauer, 1981). Although *Rhizobium* numbers in the bulk nutrient solution decreased rapidly after addition, this decrease appeared to be independent of the solution Cu^{2+} activity (Fig. 1) and may have been related to an effect of pH on cowpea *Rhizobium* survival (Keyser et al., 1979). *Rhizobium* numbers in the rhizosphere decreased only slightly between their measurement 3 DAI and 12 DAI even in the highest Cu treatment (data not presented), probably because of a pH buffering effect of the rhizosphere mucigel. Hence, it is considered that the reduction in nodulation observed in solutions containing a Cu^{2+} activity $\geq 0.77 \mu\text{M}$ represents predominantly a host plant effect (due to the inhibition of root hair growth) rather than a toxic effect of the Cu on the *Rhizobium*, although the rhizobia populations in the bulk solution declined over time.

These results observed for Cu are similar to those reported by others investigating the effect of Al stress on nodulation. Brady et al. (1990) concluded that the nodulation process in soybean is directly affected by the Al inhibition of root hair formation. Also studying Al, Alva et al. (1987) found that whilst shoot and root growth in soybean were reduced at monomeric Al activities $\geq 5\text{-}9 \mu\text{M}$, nodule formation was decreased when the Al activity $\geq 0.4 \mu\text{M}$. Similarly, it has also been shown that Al has a greater effect on the nodulation process in *Stylosanthes* spp. (due to a reduction in root hair infection sites) than on the survival of free-living *Rhizobium* (de Carvalho et al., 1981; de Carvalho et al., 1982).

Plant growth was decreased significantly with increasing Cu^{2+} activity, with shoot and root yield decreasing at Cu^{2+} activities $\geq 1.0 \mu\text{M}$ (Fig. 1). Kopittke & Menzies (2006)

also found growth of cowpea to decrease significantly at Cu^{2+} activities $\geq 1.7 \mu\text{M}$. Although nutrient supply was generally maintained in the range considered adequate (Fig. 2), the observed reduction in growth was associated with a reduction in shoot concentrations of Ca, Mg, Fe, and Zn to levels that were likely limiting to growth (data not presented). This was confirmed by the visual observations, with shoots in the two highest Cu treatments displaying interveinal chlorosis consistent with Fe deficiency. Indeed, these observations correspond well to those presented by Kopittke & Menzies (2006) who used the same experimental system as this study, and presented full tissue analysis results. Tissue concentrations of N were low, with the foliar symptoms of N deficiency observed, and the shoot concentrations (10-17 mg/g) below the critical concentration for deficiency (15-30 mg/g) (Reuter and Edwards, 1997). Shoot and root N concentrations were highest in the two largest Cu^{2+} treatments. This was expected, as no N was supplied in the nutrient solution (Table 1), and the nodules present had little time to develop and fix nitrogen. Very few nodules contained hemoglobin. The original seed N-reserves were thus diluted by plant growth, the least dilution in the two largest Cu^{2+} treatments. As previously reported for Cu^{2+} toxicity, the Cu^{2+} activity producing reduced root hair growth ($0.77 \mu\text{M}$) was smaller than that required to reduce both shoot and root growth ($1.0 \mu\text{M}$) (Fig. 1 and Fig. 4) (Kopittke and Menzies, 2006; Sheldon and Menzies, 2005).

The elevated solution Mn concentrations ($0.3\text{-}0.6 \mu\text{M}$), due to oversupply of the Mn-saturated resin, resulted in the formation of Mn toxicity in the low Cu treatments approximately 3 d before completion of the experiment (corresponding shoot Mn concentrations of $190\text{-}250 \mu\text{g/g}$). Although solution Mn concentrations were similarly

large in all treatments, due to the reduced root uptake of Mn at high Cu^{2+} activities (as discussed above for Ca, Mg, Fe, and Zn), tissue Mn concentrations in these treatments were substantially lower. The onset of Mn toxicity symptoms 13 DAP was not likely to have affected nodulation or plant growth, as Mn toxicity did not reduce root hair growth in *Trifolium repens* (Wood et al., 1984), nor in the current study where good root hair growth was observed in the low Cu solutions. Furthermore, any growth reductions due to Mn toxicity would have occurred predominantly in the lower Cu treatments, thus actually reducing differences between the treatments.

Conclusions

The nodulation of cowpea was found to be more sensitive to increasing Cu^{2+} activities than both shoot and root growth; whilst a Cu^{2+} activity of $1.0 \mu\text{M}$ corresponded to a 10 % reduction in shoot and root weight, a Cu^{2+} activity of $0.2 \mu\text{M}$ caused a 10 % reduction in nodulation. This reduction in nodulation was due to the inhibition of root hair formation, rather than to a reduction in the size of the *Rhizobium* population. Root hair growth was reduced in solutions containing a Cu^{2+} activity of $0.77 \mu\text{M}$, with few root hairs observed in solutions containing $\geq 1.0 \mu\text{M} \text{Cu}^{2+}$. Furthermore, root hairs which did form at $0.77 \mu\text{M} \text{Cu}^{2+}$ were often short, stubby and straight. The reduction in shoot growth was associated with a decrease in tissue concentrations of Ca, Mg, Fe, and Zn, whilst roots at high Cu^{2+} activities had bent tips, displayed excessive lateral branching, and were short, stubby, and often swollen behind the root tip.

Acknowledgments

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Table 1 Composition of the basal nutrient solution supplied at the beginning of the experiment, and the delivery nutrient solution added throughout the duration of the experiment in quantities calculated to meet plant demand.

	Basal	Delivery
	(μM)	(mM)
N	0	0
Cl	800	734
K	302	400
Ca	700	150
Mg	50	75
S	502	49
P	2	20
Fe	10	5
B	3	1
Mo	0.02	0.02
Cu [†]	0	0
Mn [†]	0	0
Zn [†]	0	0

[†]These nutrients were supplied by the Cu-, Mn-, and Zn-forms of the cation exchange resin

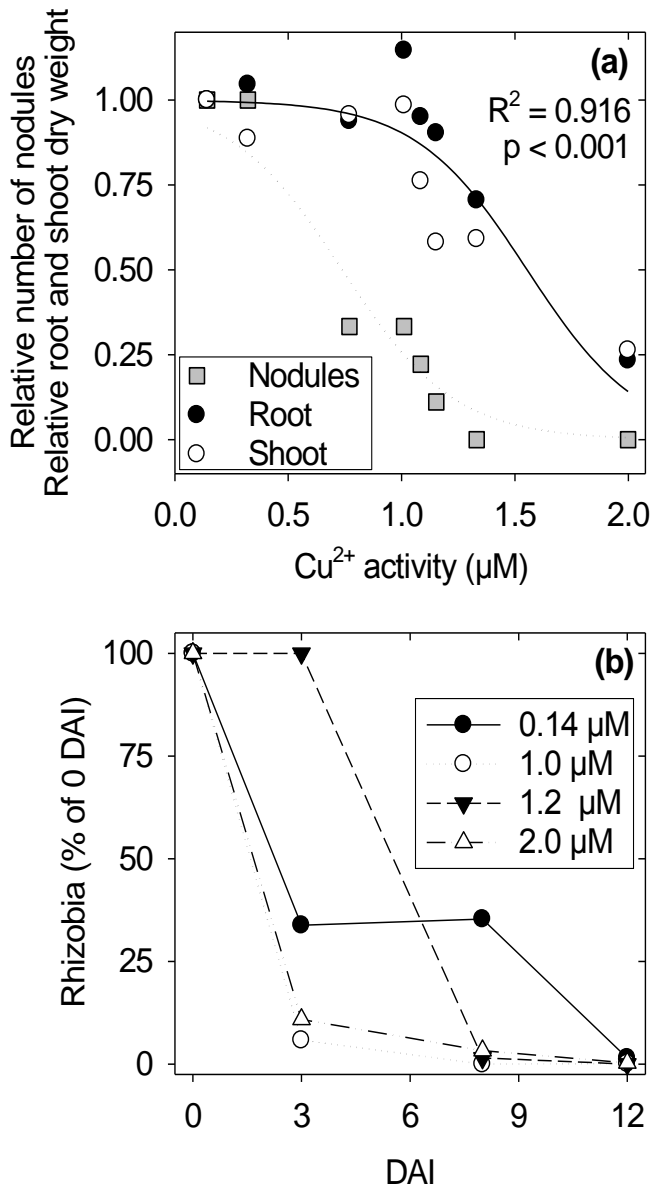


Fig. 1 Effects of solution Cu^{2+} activity in a dilute nutrient solution on the growth of cowpea (*Vigna unguiculata* (L.) Walp. cv. Caloona); (a) relative number of nodules per plant and relative dry mass of the roots and shoots after 16 d of growth, and (b) survival of the rhizobia in the bulk nutrient solution (calculated by the most probable number method) compared to that calculated 0 days after inoculation (DAI) for various Cu^{2+} activities. Results for (a) are arithmetic means of three replicates, and results for (b) are arithmetic means of two replicates. For (a): $\text{DryMass} = (e^{(6.3 - 4.0(\text{Cu}))}) / (1 + e^{(6.3 - 4.0(\text{Cu}))})$, and: $\text{Nodules} = (e^{(3.0 - 4.0(\text{Cu}))}) / (1 + e^{(3.0 - 4.0(\text{Cu}))})$.

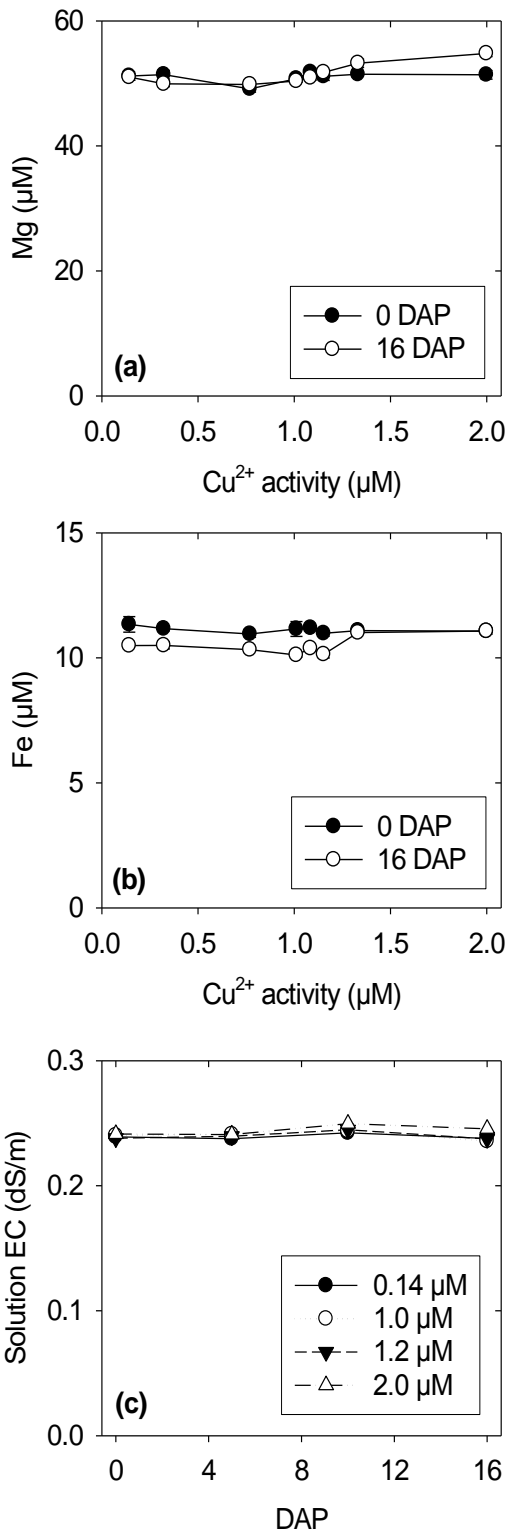


Fig. 2 Effects of solution Cu^{2+} activity on solution concentrations of (a) Mg, and (b) Fe measured 0 and 16 days after planting (DAP), and (c) changes in nutrient solution electrical conductivity (EC) at four selected Cu^{2+} activities over the duration of the experimental period. Results are arithmetic means of three replicates, bars indicate the standard error of mean.

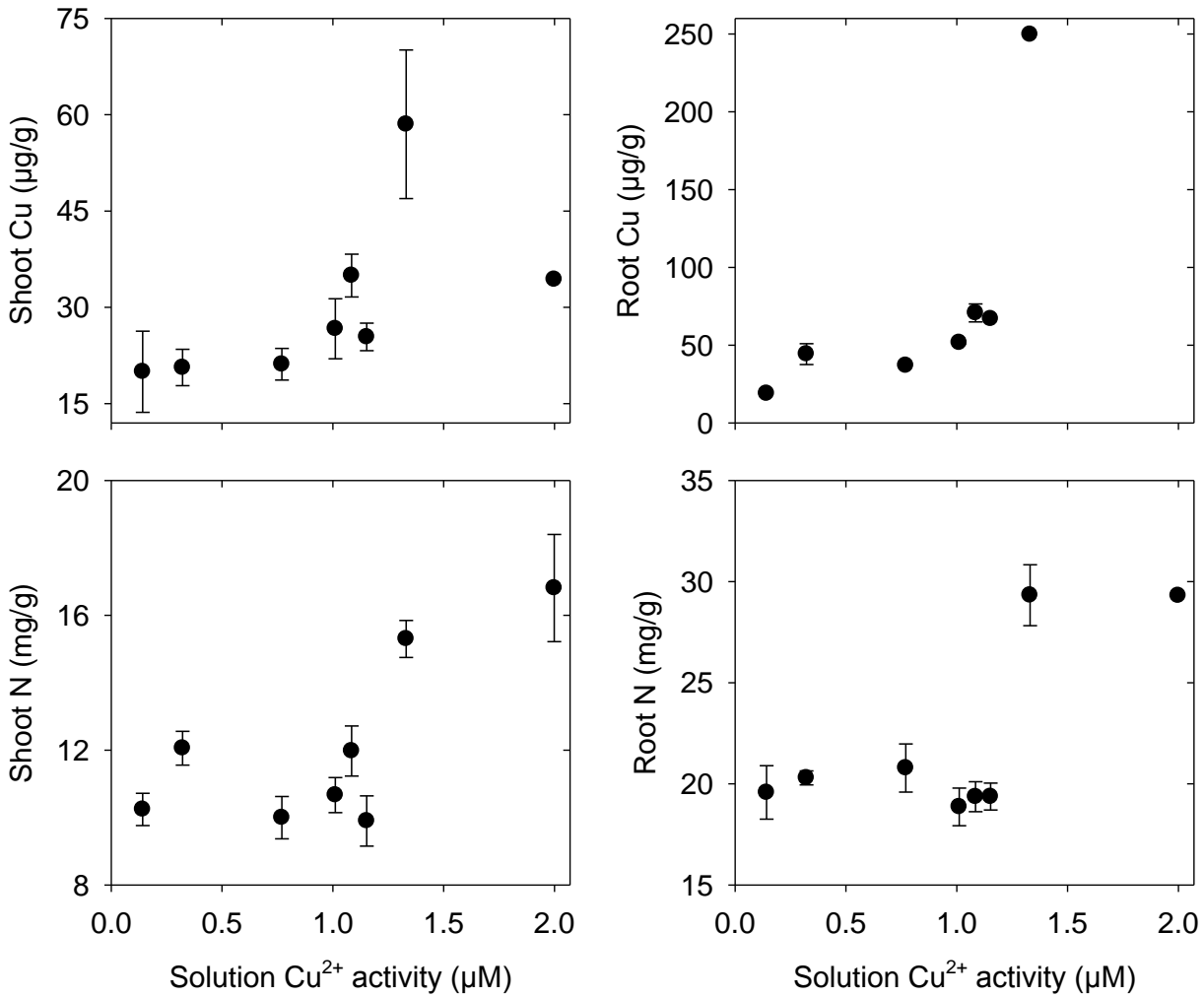


Fig. 3 Effects of solution Cu²⁺ activity in a dilute nutrient solution on the shoot (left) and root (right) tissue concentrations of Cu (top) and N (bottom) in cowpea (*Vigna unguiculata* (L.) Walp. cv. Caloona). Results are arithmetic means of three replicates, bars indicate the standard error of mean. Standard errors are not presented for some of the higher Cu²⁺ treatments where replicates were combined to give sufficient sample for analysis. There was insufficient sample for analysis of root Cu at the highest Cu²⁺ activity.

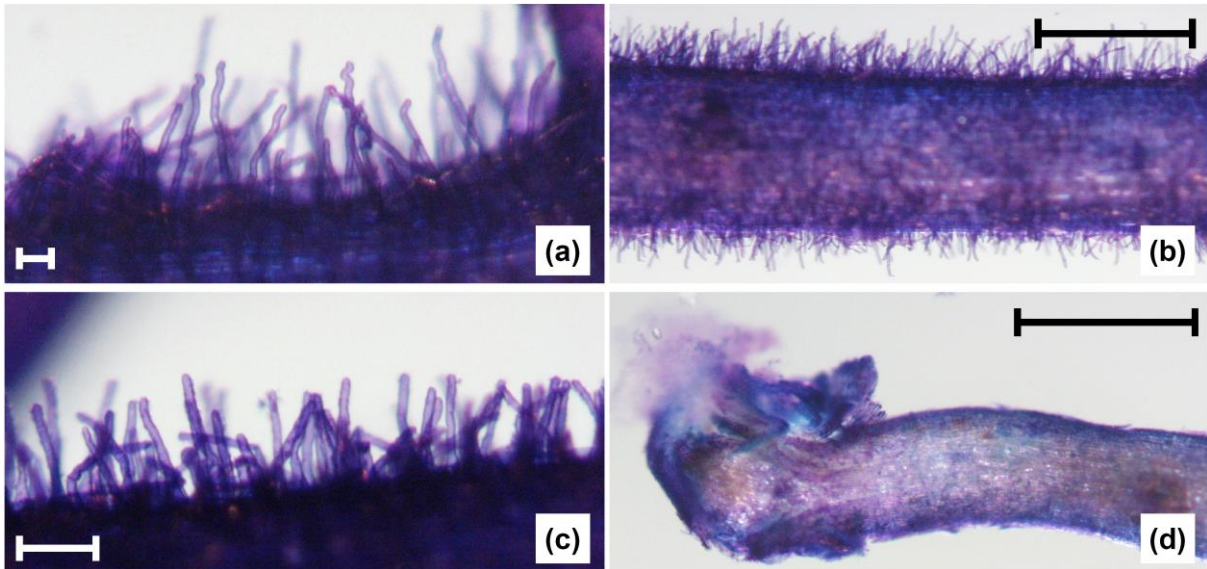


Fig. 4 Roots of cowpea (*Vigna unguiculata* (L.) Walp. cv. Caloona) stained with 0.5 % toluidine blue O after 16 d growth in a dilute nutrient solution containing various Cu^{2+} activities. Optical micrographs from roots at (a) and (b) $0.32 \mu\text{M Cu}^{2+}$, (c) $0.77 \mu\text{M Cu}^{2+}$, and (d) $1.3 \mu\text{M Cu}^{2+}$. For (b) and (d) bar indicates approximately 1 mm, whilst for (a) and (c) bar indicates approximately 0.1 mm.