Effects of elevated copper levels on biological nitrogen fixation and occurrence of rhizobia in a Tanzanian coffee-cropped soil

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Abstract: A study was conducted to investigate the effects of increasing copper concentration in soil on rhizobial occurrence and the process of biological nitrogen fixation. Two slow-growing bradyrhizobial strains CP_1 and GM_8 and two fast-growing rhizobial strains PV_1 and PV_2 were studied by comparing their performance under increasing copper concentrations in greenhouse-based assays involving modified Leonard jar assemblies and potted-soil experiments. Additionally, field samples from soils grown to coffee and subjected to long-term use of copper-based fungicides were analyzed for their total indigenous rhizobial populations using the most probable number-plant infection technique. Results indicated that elevated copper levels in the growth medium had inhibitory effects on nodulation, biological N_2 fixation and overall rhizobial numbers in soil. Significant ($p= 0.05\%$) reductions in fresh nodule mass, fresh nodule volume and total shoot nitrogen were recorded when copper concentration was increased from 0 to 100 ppm in both modified Leonard jar assemblies and potted-soil trials. Effective decrements in all the three parameters of fresh nodule mass, fresh nodule volume and total shoot nitrogen were more pronounced with the slow-growing bradyrhizobial strains of CP_1 and GM_8 than with the fast-growing PV_1 and PV_2. The MPN-plant infection technique results showed a non-significant ($p=0.05$) but substantial decrement in rhizobial and bradyrhizobial numbers when the copper-contaminated field soil (82.5 mgCu/g soil) was compared to a control soil (1.8 mgCu/g soil). The study concludes, therefore, that elevated levels of copper in soil could be harmful to free-living rhizobia and their abilities to fix N_2 in respective symbiotic associations with legume species. Such negative effects were more pronounced in the slow-growing bradyrhizobial than rhizobial species used.

Keywords: Biological nitrogen fixation; Copper contamination; Rhizobia; MPN-plant infection technique

1. Introduction

Symbiotic associations involving legume species of the flowering family *Fabaceae* and the legume- nodulating bacteria of the family *Rhizobiaceae* are well known for their ability to fix atmospheric dinitrogen gas to plant-available forms of N. Their association increases soil N levels thus facilitating plant growth and development. Legume-*Rhizobium* associations occurring in stressed environmental conditions form one of the most widely studied practical aspects of biological sciences (Ahmad et al., 2012). One of these stressful environmental conditions is contamination of soils in which they grow with heavy metals such as copper, zinc, cobalt and manganese. Although all these metals are essential for plant and microbial growth, elevated quantities are toxic as they negatively affect microbial and plant growth, nodulation and nitrogenase activity of plants (Arora et al., 2010; Ahmad et al., 2012). Rhizobial numbers, for example, have been shown to decrease with increasing concentrations of copper, zinc and lead in soils subjected to artificial pollution, (Stan et al., 2011). Certain Rhizobia species have been shown to survive heavy metal contamination but their symbiotic associations lost the ability to fix nitrogen (Broos et al 2004; Hirsch et al. 1993).

Copper contamination due to prolonged use of copper-based fungicides is a growing problem in Tanzanian agricultural soils. Use of copper-based fungicides in agricultural production has been recorded since 1956/57 and has been going on since then (Bujulu et al., 1978). Cropping systems where copper-based fungicides have been the most widely used are coffee (Bujulu et al., 1978) and vegetables (Munisi and Semu, 2001), and a number of studies have indicated that such practices often resulted in soil contamination. It was found that diethylene triamine pentaacetic acid (DTPA)-extractable copper increased from an average of 3.5 $mgkg^{-1}$ in control soils to 81 mg $Cukg^{-1}$ in soils that had received copper-based fungicides for more than 30 years (Baruti, 1997). Total and DTPA-extractable copper in agricultural soils were shown to decrease with depth indicating that most of copper accidentally applied to soils via fungicide use is retained in the upper layers of the soil (Tiluhongelwa, 1999) thus increasing the potential for its negative effects on crops and microorganisms.

Table 1: Characteristics of the soil usedCN* = Contaminated soil, CS* = Control (uncontaminated) soil.

Parameter determined	Soil from Mbozi, Tanzania	
	CN*	CS*
CEC (Cmol(+)/Kg	15.5	14.9
pH (in water)	6.5	6.5
Available N (μg/kg soil)	15.7	13.8
Bray-1 P (mg/k gsoil)	7.8	5.1
DTPA- extractable Cu (ppm)	82.5	1.8

In the current study, we have examined the occurrence of rhizobial and bradyrhizobial strains in Cu contaminated agricultural soils and compared the effect of copper contamination of such soils on population numbers of the strains in question. We report further the negative effects of elevated levels of copper on nodulation and the process of biological nitrogen fixation under screen house conditions.

2. Materials and methods

2.1 Properties of soils used

Soils used in this study were characterised with respect to parameters that could affect the overall nitrogen fixing capacities of *Rhizobia*-legume associations which in addition to DTPA-extractable Cu, included pH, cation exchange capacity (CEC), Bray I P values and available nitrogen as summarized in Table 1. The near neutral pH values needed no adjustments as biological N fixation (BNF) occurs best at near neutral reactions of the growth medium (Vincent, 1970). Phosphorus (P) levels were too low (Msanya et al., (2001) in all soils and thus soil used in potted-soil experiments were supplemented with Triple Supper Phosphate to an equivalent rate of 40 KgP/ha. The level of mineral N content in the root zone is well known to affect symbiotic BNF through its inhibitory effects on nodulation (Abdel Wahab et al., 1996; Herridge et al., 1988) and nitrogenase activity (Arora et al., 2010) as the legume uses less energy to take up the freely available N from the soil that required to fix N from the atmosphere. It is from this fact that the N content of the soil was down-regulated from 17 to 0.8% by incubating it with a wide C: N ratio carbon source- maize bran at an equivalent rate of 5 tons per hectare for six weeks to allow the microbial community to immobilize the available N in the soil prior to its use in potted soil experiments.

2.2 Bacterial strains and cultures

Two strains of the fast – growing bean rhizobia- PV_1 and PV_2 and two others of the slow – growing bradyrhizobia infecting cowpeas (CP_1) and soybean (GM_8) were previously isolated and maintained at 4°C on slants of Yeast-extract Mannitol Agar (YMA) following the procedure described by Vincent (1970). Cultures for subsequent copper toxicity experiments were initiated by inoculating the stock cultures into sterile Yeast-extract mannitol broth and incubated on an automatic shaking water bath set to 200 rpm at 28°C for 7 days. At the end of the incubation period, the number of colony forming units (CFU/ml) was determined by the Miles and Misra drop-plate procedure (Vincent, 1970).

2.3 Comparison of rhizobial and bradyrhizobial populations in Cu contaminated soils under long-term coffee monoculture

Comparison of rhizobial and bradyrhizobial population sizes was done using top soil (0-15 cm) sampled from (i) a copper-contaminated field that had been under constant copper-based fungicide use for over 30 years and (ii) an adjacent (control) field repeatedly cropped to maize for an equal period of time. The two fields were first compared with respect to their DTPA-extractable copper levels prior to a subsequent estimation of rhizobial and bradyrhizobial numbers using the most probable number (MPN)-Plant infection technique as described previously (Anderson and Ingram, 1993). The experiment was considered a 2 x 4 factorial from which ANOVA and subsequent mean separation were performed.

2.4 Nodulation and symbiotic BNF experimental design and treatments

The effect of copper concentration in the growth medium on symbiotic BNF was assessed under greenhouse conditions using two strains of bradyrhizobia-GM_8 and CP_1, nodulating soybeans and cowpeas, respectively, and two rhizobial strains PV_1 and PV_2 nodulating beans. To achieve this, two separate experiments were carried out. In an *in vitro* experiment, modified Leonard Jar technique was employed.

One ml of culture of each of the four strains was aseptically introduced into each of the four planting holes in modified Leonard jars containing a nitrogen-free nutrient solution plus the predetermined doses of $CuSO_4$ to achieve final concentrations of 0, 20, 40, 60, 80 and 100 ppm Cu. Seeds were allowed to germinate and later thinned to two healthy plants per jar. The test vessels were then placed in a screen house and left for five weeks to allow the bacteria to nodulate their host legume and fix nitrogen. Plants were then harvested and fresh nodule mass, fresh nodule volume and total (shoot) nitrogen were determined as parameters of BNF. A sensitive electronic balance was used to measure fresh nodule weights of the harvested plants. A measuring cylinder carefully cut on both ends and sealed by parafilm paper on one end was used to determine nodule fresh volumes as previously described (Msumali and Kipe – Nolt, 2002).Total nitrogen was determined using the Kjedahl wet digestion procedure as described by Anderson and Ingram (1993).

Additionally, a parallel potted soil experiment was set up to augment the *in vitro* Leonard jar experiments. Briefly, the soil whose important properties affecting BNF had been adjusted as described above was treated with predetermined amounts of $CuSO_4$ to achieve final concentrations of 0, 20, 40, 60, 80 and 100 µg Cu/g soil prior to seed sowing. Four seeds were planted per pot and inoculated with 1ml/hole of pure culture of an appropriate strain. The seeds were allowed to germinate and later thinned to 2 healthy plants per pot. The pots were placed under screen house conditions with regular watering for 35 days for the bacteria to nodulate their host legume and fix nitrogen. Plants were harvested and the three variables-nodule fresh mass, nodule fresh volume and total plant nitrogen were determined in the laboratory by procedure described above. All experiments were replicated three times and repeated twice each.

2.5 Statistical analysis

The entire study was considered to be a two - factor factorial experiment organized in a Randomized Complete Block Design (RCBD). Statistical analyses were done using both excel and Statistical Analysis Systems (SAS) version 9.2 (SAS institute Inc. Cary, NC, USA). Subsequent separation of treatment means was done according to the New Duncan's Multiple Range (DMR) test at the 0.05 probability level.

3. Results

3.1 Copper contamination of soils resulted in differential reduction in population sizes between slow- and fast-growing rhizobia

DTPA-extractable copper concentration along with other parameters of the two soils that would affect symbiotic BNF were determined as described in Anderson and Ingram (1993) and results are presented in Table 1. Subsequently, Rhizobial and bradyrhizobial numbers were estimated by the MPN-plant infection technique. Despite the significant difference in DTPA-extractable copper concentration between the contaminated field (82.5 mgCu/gsoil) and the control (1.8 mgCu/gsoil) (Table 1), the differences in MPN values of each of the four strains were non-significant ($p=0.05$). There was, however, a general reduction in MPN values in the copper contaminated soil and, in particular, bradyrhizobial strains nodulating cowpeas and soybeans exhibited an appreciable reduction compared to the control (Table 2).

3.2 Elevated levels of copper have inhibitory effects on nodulation and N_2 fixation

3.2.1 Nodulation

The effect of copper on nodulation was measured by monitoring fresh nodule volumes and fresh nodule masses both in Leonard jar and potted soil experiments. Increasing copper concentration resulted in a decrease in fresh nodule volume in each of the four symbioses between appropriate legume and strains of PV_1, PV_2, GM_8 and CP_1. However, the slow-growing bradyrhizobial strains CP_1 and GM_8 suffered more pronounced reductions with effective decrements of 88.6 and 90.3%, against 18.1 and 5.6% for PV_1 and PV_2, respectively, when 0 and 100 $mgCuL^{-1}$ treatments were compared in the *in vitro* Leonard Jar-based studies (Table 3).

Results from a parallel potted soil experiment gave a similar pattern with CP_1 and GM_8 recording higher effective decrements in fresh nodule volumes (37.3 and 39.2%, respectively) compared to those of PV_1 and PV_2 (17.2 and

Table 2: Most Probable Number of rhizobial populations in copper contaminated and uncontaminated soilsMeans in the same row followed by the same letter are not significantly (p = 0.05) different according to the New Duncan's Multiple Range Test.

Group	Rhizobia type nodulating:	Uncontaminated soil Mean MPN (CFU/g)	Contaminated soil (Mn Mean MPN (CFU/g)
Fast – growers	*Phaseolus vulgaris*	3932a	3802a
	Phaseolus vulgaris	3437a	3125a
Slow – growers	*Vigna unguiculata*	1816b	1097b
	Glycine max	1855b	1295b

Table 3. Effects of copper on nodule volume *in vitro*Means in the same column followed by the same letters are not significantly different (p = 0.05) according to the New Duncan's Multiple Range (DMR) Test.

Copper concentration (ppm)	Nodule volumes (ml/plant) under			
	PV_1	PV_2	CP_1	GM_8
00	1.200cd	1.200 cd	2.633a	1.367b
20	1.167cde	1.200cd	1.367b	0.813j
40	1.133def	1.100efg	0.600k	1.067fgh
60	1.020 ghi	1.233c	0.467l	0.833j
80	1.033ghi	0.967i	0.314m	0.333m
100	0.983hi	1.133def	0.300m	0.133n

Table 4. Effects of copper on nodule volume *in vivo*Means in the same column followed by the same letters are not significantly different (p = 0.05) according to the Duncan's Multiple Range (DMR) Test.

Copper concentration (ppm)	Nodule volumes (ml/plant) under			
	PV_1	PV_2	CP_1	GM_8
00	4.956a	4.713abcd	4.98a	4.847ab
20	4.587bcde	4.413defg	4.763abc	4.727abcd
40	4.613bcde	4.450cdef	4.787abc	4.724abcd
60	4.597bcde	4.250fg	3.763i	3.773i
80	4.283efg	4.133fgh	3.277jk	3.456j
100	4.103gh	3.857hi	3.030k	3.039k

18.2%) (Table 4).

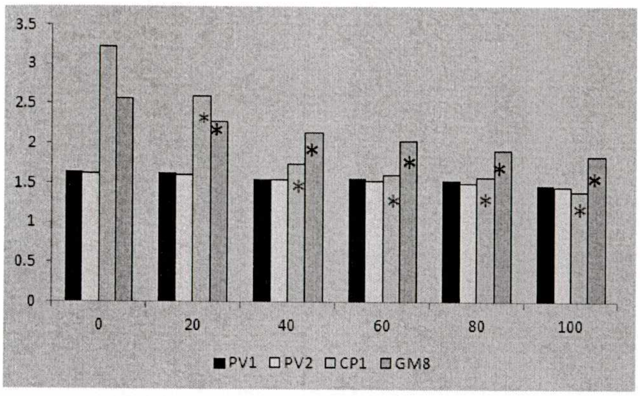
Similarly, copper affected fresh nodule masses of the fast-growers and the slow-growers differently. In the modified Leonard Jars, increasing the copper concentration from 0 to 100 $mgCuL^{-1}$ caused higher effective reductions in fresh nodule masses of CP_1 and GM_8 (94.2 and 83.7%, respectively) compared to 50.3 and 50.7% for the fast-growing PV_1 and PV_2, respectively (Table 5). The Fresh nodule mass effective decrements in the potted soil experiments were 71.4 and 60.8% for CP_1 and GM_8, compared to 19.1 and 12.2% for PV_1 and PV_2, respectively (Table 6).

Table 5: Effect of copper on nodule mass *in vitro*Means in the same column followed by the same letters are not significantly different (p = 0.05) according to the Duncan's Multiple Range (DMR) test.

Copper concentration (ppm)	Nodule mass (mg/plant) under			
	Fast – growing rhizobia		Slow – growing rhizobia	
	PV_1	PV_2	CP_1	GM_8
00	953b	953b	1131a	533i
20	803c	783d	553h	407l
40	750e	742ef	170o	343m
60	737f	713g	158o	247n
80	513j	547h	128p	170o
100	473k	470k	65r	87q

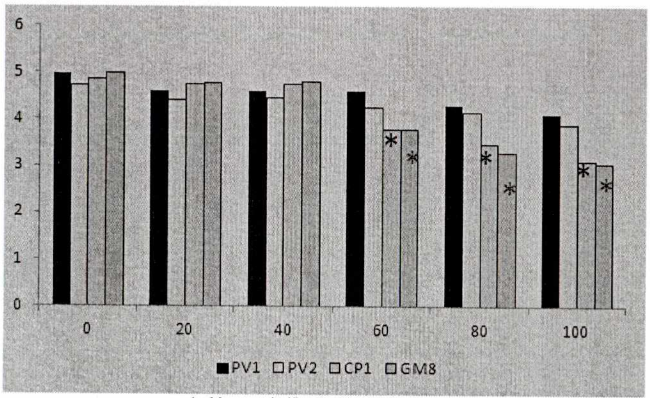
Table 6: Effects of copper on nodule mass *in vivo*Means in the same column followed by the same letters are not significantly different (p = 0.05) based on the Duncan's Multiple Range (DMR) Test

Copper concentration (ppm)	Nodule mass (mg/plant) under			
	Fast-growing rhizobia		Slow – growing rhizobia	
	PV_1	PV_2	CP_1	GM_8
0	577k	467o	1610a	1172c
20	547l	467o	1607b	1159d
40	490n	467o	1073f	1110e
60	503m	437r	920h	953g
80	457q	430s	890j	893i
100	467o	410t	460p	460p



* = Means are significantly (P= 0.05) different from the control

Figure1. Effect of copper concentration on total shoot nitrogen (*in vitro* condition).



* = Means are significantly (P= 0.05) different from the control

Figure 2. Effect of copper concentration on total shoot nitrogen (*in vivo* condition).

4. 3.2.1 Total N content

Total Nitrogen content of the plant was used in this study to estimate the N_2 fixing capacity of the relevant symbioses. Increasing copper concentration resulted in reduced total shoot nitrogen both *in vitro* (Leonard jar experiment) and *in vivo* (potted soil). A significant reduction in total shoot nitrogen was observed when copper was increased from 0 to 100 mgCuL^{-1}*in vitros* (Figure 1) and *in vivo* (Figure 2). Consistently, the slow-growing bradyrhizobial strains of CP_1 and GM_8 exhibited a far greater reduction with effective decrements of 28.8 and 56.9%, compared to 10.4 and 10.5% for PV_1 and PV_2 respectively when 0 and 100 mgCuL^{-1} were taken into account in modified Leonard Jar-based studies. A similar pattern was observed in the potted soil-based studies where increasing copper concentration from 0 to 100 mg.g^{-1} resulted in effective decrements in total shoot nitrogen of 39.2 and 36.2% for GM_8 and CP_1 compared to 17.2 and 18.2% for PV_1 and PV_2 respectively (Figure 2).

5. Discussion

Several studies have shown that symbiotic BNF is sensitive to abiotic stresses and, in particular, excessive levels of trace metals in the growth medium. Adverse effects of excessive copper concentrations on symbiotic BNF may range from inhibitory growth effects on the microsymbiont (Stan et al., 2011) to nodule growth, development and functioning (Carpena et al., 2003; Sanchez-Padro et al., 2012). In the present study, the effects of elevated copper levels on the occurrence of free slow-growing bradyrhizobial and fast-growing rhizobial populations in the field and its effects on BNF under screen-house conditions were examined. Copper affected both groups but more adversely on the slow-growing bradyrhizobial strains than the fast-growing rhizobia. Several reports have shown that increasing copper concentration leads to a reduction in numbers of free-living (brady) rhizobia in soils. Stan et al., (2011), reported a relative decrease in rhizobial numbers with increasing copper concentration artificially applied to a field plot grown to red clover plants. Similarly, Chaudry et al., (2000) reported a reduction in rhizobia numbers especially when copper concentrations reached values > 250 mg K^{-1} soil.

Copper accumulation in growth media has been reported to interfere with nodulation and the overall N_2 fixing potential of rhizobia-legume associations. It was observed in the present study that increasing copper concentrations in the growth medium (either N-free nutrient solution or potted soil) led to a decrease in two nodulation parameters, fresh nodule mass and volume. Consistently, the reductions were more pronounced in the symbiotic associations involving the slow-growing strain of CP_1 and GM_8 than those of the fast-growing PV_1 and PV_2. The inhibitory effects of copper on nodulation and N_2 fixation have been attributed to its accumulation on the inner cortex and infected zones of the nodule thereby inducing abnormalities in the structure and utrastructure of the nodules (Sánchez-Pardo et al., 2012). The effects in turn, culminate in the observable reductions in a variety of nodulation parameters including nodule numbers, fresh or dry nodule weight and nodule volumes. In this respect, significant reductions in nodule dry weight per plant were reported when copper concentration was increased to 100 ppm (Bhandal et al., 1989). Similarly, copper concentration of 192µM in N-free nutrient solution was reported to reduce nodule weight along with nodule number and total plant nitrogen in both *Lupinus albus* L. and *Glycine max* L. (Sánchez-Pardo et al., 2012).

Results in the current study suggested that slow-growing bradyrhizobia either in their free-living state or in appropriate symbiotic associations were more susceptible to copper toxicity than the fast-growers. Although the molecular basis remains unknown, similar observations have been reported previously (Aroa et al., 2010). In their study, a bradyrhizobium strain BMP_1 was found to be more susceptible to metal stresses caused by Al, Cu, Fe and Mo than the relatively fast-growing *Sinorhizobium meliloti* under explanta conditions. In particular, unlike in *S. meliloti* strain RMP_5, Cu showed inhibitory effects on growth and enzymatic activities of BMP_1 at all concentrations tested (0.1-100mM).

6. Conclusions

In conclusion, elevated levels of copper in the growth media were shown in the present study to be harmful to both the free-living rhizobia and their corresponding symbiotic associations with the legumes. The study has shown that the slow-growing bradyrhizobial strains of CP_1 and GM_8 were clearly more susceptible to elevated levels of copper in the growth medium than the fast-growing PV_1 and PV_2. As noted above much remains to be done to supplement the observations made in this and a few other studies and more importantly establish the molecular basis for the apparent differential response to copper toxicity between the fast- and slow-growing strains of legume-nodulating rhizobia

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