EFFECTS OF COPPER CONCENTRATION IN SOIL ON PROLIFERATION AND SURVIVAL OF RHIZOBIA, NODULATION AND BIOLOGICAL NITROGEN FIXATION IN BEANS, COWPEAS AND SOYBEANS

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ABSTRACT

A study was conducted in which two strains of the fast – growing bean rhizobia- PV_1 and PV_2 and two others of the slow – growing bradyrhizobia for cowpeas and soybean- CP_1 & GM_8, respectively, were used to test their ability to: - (a) proliferate in copper contaminated liquid media, (b) survive in copper contaminated soil, (c) nodulate respective host legumes and (d) fix nitrogen under increasing copper levels both *in vitro* and *in vivo*. Known population sizes of each of the strains were exposed to copper concentrations in the range: 0, 20, 40 60, 80 and 100 ppm in either Yeast - extract Mannitol Broth or in modified Leonard Jar assemblies or potted soil. Most Probable Number (MPN) studies were also done to estimate populations of the native strains of the fast - and slow - growing rhizobia in a Cu -contaminated soil. Results indicated that copper was more toxic to the slow – growing bradyrhizobia than to the fast – growing rhizobia. Reduction in population sizes *in vitro* was more significant ($p = 0.05$) for bradyrhizobial than for rhizobial strains. Reductions in fresh nodule volume, fresh nodule mass and total shoot nitrogen were more significant ($p = 0.05$) in the bradyrhizobial – than in rhizobial – legume associations. Copper depressed the populations of slow – growing strain CP_1 and GM_8 *in vivo* more than those of the fast – growing strains PV_1 and PV_2. There was no statistically significant ($p = 0.05$) effect of 82.5 mg Cu/kg soil on numbers (MPN) of native rhizobial strains, probably due to adsorption of Cu by soil colloids. This study showed that based on the solution culture, potted soil and soil survival experiments, slow - growing rhizobia were more susceptible to Cu toxicity than were the fast - growing rhizobia.

DECLARATION

I, Tindwa, Hamisi, declare to the Senate of Sokoine University of Agriculture that this is my original work and that it has neither been submitted nor being concurrently submitted for degree award in any other institution.

…… ……..

Signature (Student) **Date**

…… ……

Signature (Supervisor) **Date**

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CHAPTER ONE

INTRODUCTION

1.1 General Overview of the Subject

Accumulation of copper in soils can result in from four main factors, namely (a) decomposition or weathering of naturally occurring copper bearing rocks and minerals, (b) mining and smelting of copper by man, (c) land disposal of copper containing wastes and (d) use of copper pesticides in agricultural production. Although there are known copper deposits spread across the country (Harris, 1981), there are no large – scale copper mining and smelting activities in Tanzania. However, use of copper pesticides is the main source of copper contamination in many Tanzanian agricultural soils, and such pesticides could affect useful microorganisms in such soils.

The use of copper fungicides in Tanzania dates far back in the colonial time. Use of copper fungicides in the control of fungal diseases of coffee, for example, started sometimes back in 1956/57 and has been going on since then (Bujulu *et al.*, 1978; Okioga, 1978; Mzimba, 2001). Over the period, formulations containing 50% copper and phenyl mercuric acetate were found to be the most effective fungicides. According to Lyamungo Agricultural Research Institute (1990) report, the fungicides in the coffee growing areas of Tanzania are applied at rates ranging from 5-11 $kgha^{-1}$, with a minimum of eight applications per season.

Similarly, fungal diseases have threatened the continued cultivation of vegetables in major producing areas of the country, especially during rainy periods. Thus, to control such diseases and sustain production, fungicide use has been indispensable. Copper compounds have been some of the most widely-used fungicide in vegetable production (Munisi and Semu, 2001).

Concerns have been raised, however, regarding the use of copper fungicides, due to their potential to cause phytotoxic plant damage and toxicity to beneficial microorganisms (Huysman *et al.,* 1994). Dumestre *et al.* (1999) reported reduced biological activity due to copper toxicity resulting from elevated copper concentration in soils. Baijukya (1996) reported inhibition of nodulation by Kocide 101 (77% cupric hydroxide), when applied two to four times the recommended rates, mainly due to the direct effect of the fungicide on the bean plant. Maliszewska *et al.* (1985) demonstrated in a separate study that low doses of copper (10-100 $\mu g g^{-1}$) added to sandy and alluvial soils showed a markedly harmful influence on nitrification and on the growth of bacteria and actinomycetes in both soils. It was noted, however, that there existed species specificity in their response to copper as exemplified by *Thiobacillus ferrooxidans* used in microbial leaching of copper from flotation tailings, which produced leachates containing 290-470 mg Cu/l (Lewis, 1990). In general, bacteria are more sensitive to copper than fungi (Huysman *et al.,* 1994).

Copper has also been reported to be toxic to plants. Use of copper-containing fungicides can, for example, increase soil copper levels enough to cause some plant damage. Beeson and Newton (1992) reported less growth in terms of trunk diameter of sweet gum (*Liquidambar styraciflua*) and weeping willow (*Salix chrysocoma*) grown in copper-treated pots than those grown in untreated pots. It was noted also that plants differed in their ability to tolerate copper toxicity. In a study that compared Cu toxicity in Australian trees, Mitchell *et al.* (1988) reported that concentrations of Cu in the soil found to reduce growth by 50% were 205 mg kg^{-1} for she-oak tree (*Casuarina distyla),* 560 mg kg^{-1} for yellow bloodwood (*Eucalyptus eximia*) and 610 mg kg^{-1} for heath banksia (*Banksia ericifolia*).

Numerous studies in temperate soils have shown that the use of metal – containing chemicals, zinc in particular, caused toxicity to rhizobia. However, temperate and tropical BNF systems differ with respect to soil type and legume species grown. Unlike most temperate species which form symbioses with fast-growing rhizobial genera, warm-weather crops such as *Vigna unguiculata* (cowpea) form symbioses with slow-growing rhizobia of the genus *Bradyrhizobium*; and both the macro - and the micro - symbionts of the tropics may differ in their tolerance to copper compared to temperate species. Furthermore, compared to temperate soils, tropical soils can be very different in their physico-chemical characteristics; for example, they may carry a variable charge which means that copper could exhibit different behaviour, bioavailability and, therefore, be more or less toxic than in the temperate situation.

1.2 Problem Analysis and Justification

There is evidence that symbiotic BNF can suffer from environmental stress which may reduce the survival or rate of growth of the microorganisms in their free-living state, interfere with the process of plant infection or nodule development or affect the fixation of N_2 once the symbiosis has been established (Giller and Wilson, 1991). One of those stresses could be presented by Cu or other toxic heavy metals present in or added to soils.

Fisher and Hayes (1981), for example, found that N_2 fixation appeared to be reduced mainly where the vigour and growth of the plant was inhibited by fungicides. Baijukya (1996) indicated that the impaired nodulation and low N_2 fixation when Kocide 101 (77% cupric hydroxide) was used at concentrations higher than the recommended rate was due to its adverse effects on the bean plant rather than on rhizobia.

It appears evident, therefore, that elevated amounts of soil copper have negative effects on the growth of both the macrosymbiont and microsymbiont on the one hand and, possibly, on the process of symbiotic biological nitrogen fixation, on the other. This supposition needs to be unequivocally established.

1.3 Objectives of the Study

In view of the above problem analysis and justification, a study was undertaken to examine the effects of high levels of soil copper on the proliferation and survival of rhizobia, legume growth and the process of symbiotic Biological Nitrogen Fixation (BNF). This general objective was to be realized upon achievement of three specific objectives as enlisted below:

i. To determine Cu levels that cause lethal effects to rhizobia cells *in vitro* and *in vivo*,
ii. To determine inhibitory levels of copper on nodulation of compatible legume plants and the process of symbiotic legume/rhizobial BNF *in vitro* and *in vivo*,
iii. To compare the tolerance of rhizobial and bradyrhizobial strains, and possibly populations, to elevated copper levels as can be reflected in Cu- contaminated soils compared with those in uncontaminated (adjacent) soils.

1.4 Hypotheses of the Study

The objectives above were articulated from the following hypotheses:

Hypothesis for objective 1: High Cu levels in soil do not have any lethal effects on rhizobia.

Hypothesis for objective 2: High Cu levels in soil do not inhibit nodulation and BNF.

Hypothesis for objective 3: High Cu levels in soil do not exert differential effects on growth and multiplication of different rhizobia strains.

CHAPTER TWO
LITERATURE REVIEW

2.1 Preamble

For many poor farmers BNF is a viable and cost-effective alternative solution to industrially-manufactured N fertilizers. The need for BNF improvement and its widespread application in agriculture has never been more urgent than it is today, especially for the improvement of the most vulnerable cropping systems in developing countries (Russelle and Birr, 2004). A substantial amount of work has already been done on biological nitrogen fixation on the one hand and copper toxicity to soil microorganisms in general, on the other. However, the influence of copper toxicity on symbiotic BNF in particular has not been as widely documented. If present in soil, copper may exert toxicity that could negatively impact on BNF, a situation that should be avoided whenever possible.

Although an in-depth elucidation on copper toxicity to all soil microorganisms is not within the purview of the current study, a review of such areas like copper as a necessary trace element by plants, copper accumulation in soils and copper toxicity to plants and soil microorganisms is worthwhile. Of equal significance are importance and availability of N to plants, effects of copper on biological nitrogen fixation and on rhizobia/bradyrhizobia specifically, sources of copper in soils, environmental factors constraining biological nitrogen fixation, mechanisms of copper toxicity to soil microorganisms generally and on rhizobia specifically Hence, the current study addresses *effects of copper accumulation in soil on proliferation and survival of rhizobia, legume growth and the process of biological nitrogen fixation.*

2.2 Importance and Availability of Nitrogen to Plants

Nitrogen is the most limiting nutrient for plant growth. Nitrogen makes the largest proportion (Approximately 79%) of the air. However, in the air nitrogen exists in a form directly non-usable by plants. According to Evers (2002) it is not the plant that removes nitrogen from the air but the bacteria, called rhizobia, which live in small tumour-like structures called nodules on the legume plant roots. These bacteria can take nitrogen gas from the air in the soil and transform it into ammonia (NH_3) that converts to ammonium (NH_4^+) which can then be used by the plant.

The quantity of nitrogen fixed by legumes-rhizobial symbioses can range from almost none to over 178kg/ha, accounting for about 40 to 70% of the total global nitrogen input into the soil (Keya, 1985). Factors that influence the quantity of nitrogen fixed are the level of soil nitrogen, the rhizobia strain infecting the legume, extent of legume plant growth, how the legume is managed, and length of the growing season to mention but a few (Burdass, 2002). If given a choice, a legume plant will remove the nitrogen already present in the soil before obtaining nitrogen from the air through N_2-fixation, (Evers, 2002). A legume growing on a sandy soil very low in nitrogen will get most of its nitrogen from fixing the N_2 of the air while a legume growing on a fertile soil will get most of its nitrogen from the soil (Evers, 2002). This is probably because it is natural for legume – rhizobia associations to go for a cheaper (in terms of energy and time) and readily available sources of nitrogen before a second alternative is sought.

2.3 *Rhizobium/Bradyrhizobium* and N_2 Fixation

2.3.1 Environmental factors constraining symbiotic biological N_2 fixation

It is important to strive to control the environmental factors that negatively impact on

the symbiotic biological nitrogen fixation in order to derive the benefits of nitrogen fixation. Such constraints have been extensively reviewed elsewhere, and therefore, a brief mention of them is done here to avoid unnecessary digression from the main theme of the current study.

According to Giller and Wilson (1991), the main environmental stresses can be grouped into two, namely: physical factors- notably temperature and moisture, and chemical imbalances in the growth medium. Being mesophiles, most rhizobia will exhibit optimum growth at temperatures between 25^{o} C and 35^{o}C. Thus, temperatures below 25^{o}C or above 35^{o}C will impact negatively on the survival and function of rhizobia, which in turn affects nitrogen fixation (Alexander, 1961). Symbiotic legume associations are also known to be sensitive to drought stress. Studies by Sprent (as reviewed in Bergersen, 1975) revealed that severe stress causes irreversible damage to the enzyme nitrogenase and its functions, thus depressing nitrogen fixation.

Chemical imbalances that affect nitrogen fixation can either be nutritional deficiencies or toxicities. The most common nutritional imbalances in addition to oversupply of N, include those of insufficient supply of phosphorus, cobalt, manganese, copper and molybdenum (FAO, 1983; Giller and Wilson, 1991). Chemical toxicities can either take the form of excessive acidity and aluminium toxicity, excessive alkalinity or excessive supply of essential elements such as N, Cu and Zn (Giller and Wilson, 1991). A report by Izaguirre-Mayoral and Sinclair (2004) revealed that manganese at a concentration of 0.3 µM can result in reduction of growth and nodulation in soybeans. In an *in vitro* study, Nweke *et al.* (2007) observed near 100% inhibition of nitrogenase activity by cobalt, cadmium, iron, mercury and nickel at concentrations of 0.6, 0.8, 1.2, 0.12 and 12 mM respectively. Therefore, before assessing effects of copper on nitrogen fixation by

legumes, it is important that all the negative influences mentioned above are controlled to fully realise the potential of nitrogen fixation, on which the effects of copper can then be assessed.

2.4 Sources, Contents and Plant-availability of Copper in Soils

2.4.1 Sources of copper in soils

The copper found in soils may due to a variety of sources: 1. Decomposition or weathering of naturally occurring copper bearing rocks and minerals, 2. Copper mining and smelting activities by man, 3. Use of pesticides in agricultural soils, and 4. Land disposal of copper - containing wastes.

The most common source of copper is the mineral chalcopyrite **(**$CuFeS_2$) which has about 34.5% Cu. About 50% of all copper produced world-wide is extracted from this ore. Other principle copper bearing minerals include chalcocite (Cu_2S) containing up to 79.8% Cu, Azurite ($2CuCO_3$ $Cu(OH)_2$ – 55.1% Cu, Covellite (CuS) – 66.5% Cu, Cuprite (Cu_2O) – 88.8% Cu, Bornite ($2Cu_2S$ CuS FeS) – 63.3% Cu and Malachite ($CuCO_3$ $Cu(OH)_2$- 57.3% Cu (Duckeck, 2010). Soils overlaying these deposits may have elevated levels of copper as compared to other soils.

Sites with naturally occurring copper bearing minerals have been identified in Tanzania, and they include, Nyamori Hills in Kigoma with deposits of chalcopyrite, malachite and azurite; Mpanda area; Western Chunya; and Bulongwa area in Eastern Livingstone mountains (Harris, 1981). Other chalcopyrite and malachite deposists are found at Kigugwe area near Chimala in Mbeya, on the Iramba plateau, at Makuyuni in Arusha, in Mpwapwa and Kilosa districts, at Ntaka hills in Nachingwea and around western Uluguru mountains in Morogoro (Harris, 1981).

The other source of copper and its accumulation in soils is the mining and smelting activities by man. Copper mining and smelting involves an eventual disposal of copper floatation tailings and wastewaters on the surrounding soil. According to Total Systems International (2006) copper in soils can be a result of dumping of wastewaters and solid wastes including those of electronics plating, wire drawing, copper polishing, paint manufacturing, wood preservatives, printed circuit board manufacturing, and printing operations.

Pesticide use is another source of copper and its accumulation in soils. Chiroma *et al.* (2010) found that continuous use of copper pesticides resulted in accumulation of the metal in soil and eventually in spinach grown on it. Copper compounds have been some of the most widely used fungicides, especially in vegetable and coffee production. In Tanzania levels of up to 81 mg Cu/kg (DTPA – extractable) have been found in soils that received copper fungicides for more than 30 years as compared to only 3.5 mg Cu/kg in control soils (Baruti, 1997). Munisi and Semu (2001) reported, from a study in Hai district, an accumulation of up to 459.2 mg/kg Cu in soils that had received copper for 15 – 30 years as compared to only 33.3 mg/kg Cu in control soils.

Since there are no large – scale copper mining and smelting activities in Tanzania, the main source of copper in many Tanzanian agricultural soils is, therefore, the use of copper pesticides. Copper accumulation in such soils can affect microorganisms in those soils, including N_2 fixing rhizobia since legume crops are also rotationally grown in vegetable or coffee soils that receive copper fungicides.

2.4.2 Soil content and availability of copper to plants

The copper content of soils ranges from 2 to 100 ppm with its average estimated at 30 ppm (Lindsay, 1979). Availability of copper to plants is governed primarily by the total amount of the element in soils as well as prevailing soil conditions. The availability of copper decreases slowly with increasing pH but the nature of interaction is not completely understood (Lindsay, 1979). Cu^{2+} is the most common plant available form of copper in soils. However, this ion is held so tenaciously on the colloidal surfaces, notably clay and organic matter, and this may significantly reduce its bioavailability (Schulte and Kelling, 1999). Due to the strong chelation properties of soil organic matter, copper is known to be held tenaciously by organic matter perhaps more than is any other micronutrient and, thus, its bioavailability is normally decreased in soils with high clay and/or organic matter contents (Schulte and Kelling, 1999).

Other plant nutrients are known to influence copper uptake and utilization by plants. Elevated levels of phosphorus, for example, reduce the concentration of Cu in the roots and leaves of plants, and heavy phosphate fertilization can induce Cu deficiency in plants. High levels of Fe and Zn induce copper deficiency in plants. Conversely, high levels of Cu in soils can also induce deficiency symptoms of Fe and Zn to plants (Giller and Wilson, 1991). Plants, however, vary in their requirements for copper and other trace elements.Blue and Malik (1986) noted, for example, that with white clover *(Trifolium repens*), there was yield depression in the third harvest following an initial copper application.

Oversupply of Cu in soils has to be avoided as this can negatively impact on not only the legume plant growth, but also on survival, proliferation and functioning of rhizobia.

2.5 Copper Accumulation in Soils

'The worldwide use of copper-based fungicides has resulted in copper accumulations in some agricultural soils far in excess of the amounts that are required for healthy plant growth, and numerous studies have indicated that prolonged use of copper-based chemicals often resulted in soil contamination' (Jankiewicz *et al.,* 1998). In a study by Baruti (1997), DTPA-extractable copper had increased from an average of 3.5 mg $Cukg^{-1}$ in control soils to 81 mg $Cukg^{-1}$ in soils that had received copper-based fungicides for more than 30 years. Tiluhongelwa (1999) demonstrated that both total and DTPA-extractable copper levels decreased with depth indicating that most of the copper applied to soils via fungicide use was retained and, therefore, expressed toxicity effects on the surface soil. Despite its environmental and agricultural importance, the concentration, distribution and fractionation of both anthropogenic and naturally occurring copper in soil is poorly known (Jankiewicz *et al.,* 1998).

2.6 Toxicity of Copper to Plants

In most plants tissues, the range of copper concentration is 5-15 ppm (Bowen, 1966) and in crop plants the usual range is 5 - 20 ppm (Jarvis, 1978). Geraldson *et al.* (1973) reported that the common copper content found in plant tissue of various vegetable crops was 1-40 ppm and for the bush bean, *Phaseolus vulgaris*, the concentration was between 15 and 30 ppm. According to Landon (1991) perchloric acid-extractable copper concentrations of above 100 mg/kg soil have been considered to be high for many crops.

Physiologically, copper occurs in plants as Cu^{+2} and Cu^{+}, enabling it to take part in a number of important redox reactions such as mitochondrial respiration, photosynthetic electron transport, cell wall metabolism and as cofactors in many enzymes, including cytochrome c oxidase and superoxide dismutase (SOD) (Marshner, 1995; Yruela, 2005).

However, the redox reactions that make Cu an essential element can also contribute to its inherent toxicity when in high levels, through its catalytic role leading to the production of the highly toxic hydroxyl radicals (Yruela, 2005). Such biochemical mechanisms could result in gross effects to plant growth, as examples discussed below show.

The effects of excessive copper supply to plants have been studied widely especially on economically valued plants like wheat, maize, tomato, spinach, rice and beans (Panou-filotheou *et al.*, 2001). Studying the effect of excess copper on Oregano (*Origanum vulgare* subsp. *hirtum*), an aromatic plant of high commercial value in Greece, Panou-filotheou *et al.* (2001) found that excessive copper concentration in the growth medium caused a severe reduction of stem height and root volume. Marschner (1995) observed in plants growing on copper contaminated medium that very little copper was translocated to shoots and a greater proportion tended to accumulate in root tissues suggesting that the principal effect of copper toxicity was on root growth. Sheldon and Menzies (2004) observed, in this regard, that an external Cu concentration of 0.2µM caused damage to roots of Rhodes grass (*Chloris gayana*) growing in a solution culture, with symptoms ranging from disruption of the root cuticle and reduced root hair proliferation, to severe deformation of root structure. Other workers have found evidence, however, that copper toxicity can also affect higher parts of the plant. Studies on *Crassula helmsii*, a copper hyper- accumulating plant, revealed that excessive copper concentration induces inhibition of photosynthesis mainly due to its effect on the phototosystem II (PSII) reaction centre (Kupper *et al.,* 2009). In another study, Johansson *et al*. (2005) observed that while *Pistacia terebinthus* and *Cistus creticus* accumulated most of the excess copper in the roots, *Bosea cypria* accumulated most of the Cu in the leaves. In this regard, *Knezek* (1997) noted that a large copper supply usually inhibited root growth before shoot growth, but that should not be taken to mean that roots were more sensitive to high copper

concentrations than were shoots. Rather, roots are the sites of preferential copper accumulation when the external copper supply is large.

While plants can succumb to copper toxicity, with consequences on growth and production as discussed above, they can also tolerate some levels of it. Several mechanisms of tolerance to copper toxicity in higher plants have been postulated and they include: (a) Reduced uptake into the cytosol by entrapment into the apoplastic space (b) immobilization of copper in cell walls, (c) chelation of the metals within the cytosol by a range of ligands into soluble or insoluble complexes and (d) enzyme adaptation (Knezek, 1997; Hall, 2001).

There are, however, marked differences in copper tolerance among plant species. The bush bean, for example, is much more tolerant than maize, and these differences are directly related to copper content of the shoots (Tu, 1993). Tu (1993) reported in a different study a rice yield reduction of 15% when copper was added at a rate of 60, 100 and 130 mg Cu/kg soil in acid, neutral and calcareous soils respectively. When maintained at 10 µM (=0.6 ppm) Cu^{2+} in the nutrient solution, the amphibious water plant *Crassula helmsii* has been observed to accumulate up to 9000 ppm Cu (Kupper *et al.*, 2009), an expression of its tolerance to high copper levels in its tissues.

2.7 Toxicity of Copper to Microorganisms

2.7.1 Effects of copper toxicity on microorganisms generally

Copper is an essential element and required by all organisms. However, elevated concentrations of copper are toxic to all forms of life (Macomber and Imlay, 2009). A wide variety of microorganisms have been identified as being negatively affected by elevated levels of copper. Albinus *et al*. (2005), for example, observed that exposure of

microorganisms such as bacteria, actinomycetes and fungi to copper acetate resulted in a significant decrease of their counts. They found that bacterial counts after treatment with copper were reduced to only between 2.35 - 4.94% of their counts in the control. In their study, Albinus *et al*. (2005) pointed out that the impact of heavy metal (copper, zinc and lead) acetates on microbial groups was related to both metal concentration and properties of the soil as well as to the biological peculiarities of different groups of microbes.

The effects of copper toxicity have been most extensively studied in bacteria as a group and a few of such studies are reviewed here. Cheng and Chu (2009) found that copper had deleterious effects on both *in vitro* multiplication and *in vivo* survival of *Ralstonia solanacearum*, a bacterium that causes bacterial wilt disease in tomato. They indicated that when survival was studied *in vivo* (in sand as a medium), the effects were negative but not as severe as when studied *in vitro* (in liquid medium). Copper has also been shown to have dramatic bactericidal effect to *Mycobacterium tuberculosis*, the bacterium that causes tuberculosis to humans. Ward *et al.* (2008) observed that after two weeks of incubation at a concentration of 500 μM, the population size of *Mycobacterium tuberculosis* decreased 100-fold in the number of Colony Forming Units (CFU) compared with the initial inoculum. Rajesh *et al.* (2001) observed that Cu (II) was toxic to a sulphur reducing bacteria, *Desulfovibrio desulfuricans.*

The toxicity effects were observed in terms of inhibition in total cell protein, longer lag times, lower specific growth rates, and in some cases no measurable growth. It has also been demonstrated that the highly toxic *E. coli* O157:H7 strain of bacteria survived for much shorter periods of time on copper and brass surfaces than on stainless steel (Bill, 2000). Vardanyan and Trchounian (2010) suggested that excess copper inhibited cell growth of the bacterium *Enterococcus hirae* by directly inducing conformational changes

in the proton translocating protein complex called F(o)F(1) ATPase, leading to the decrease in its activity that subsequently led to inhibition of cell growth.

The effect of excessive copper exposure has also been studied in viruses. Gadi *et al*. (2008) observed that copper (in terms of filters containing copper oxide powder) can efficiently and quickly inactivate the Human Immunodeficiency Virus Type 1 (HIV-1). They observed that only a few minutes of exposure were enough to render both free viruses and virions being formed within the cytoplasm of host cells non-infectious.

2.7.2 General effects of high copper levels on rhizobia

Some work on toxic effects of copper has also been done specifically on rhizobia. Alexander *et al*. (1999), for example, reported that high copper levels affected growth of *Rhizobium leguminosarum* (and that of another bacterium, *Agrobacter tumefaciens)*, by inducing a Viable But Non Culturable (VBNC) state of their cells. Younis (2007) reported from a pot experiment that nodule numbers and mass of a *Lablab purpureus*- rhizobia association were enhanced when the soil was treated with up to 100 ppm of Co and Cu but significant decreases in numbers and mass were observed when the concentrations were raised to 150 and 200 ppm of Cu and Co, respectively, and this was attributed to severe reductions in protein content of both nodule cytosol and bacteroid fractions and leghaemoglobin concentration of nodule cytosol. Nie *et al.* (2002) demonstrated that there existed differences in the ability to tolerate copper toxicity among rhizobia – legume associations. They observed that the *Rhizobium* - *Acacia auriculaeformis* association had a stronger tolerance to Cu^{2+} ion than the *Rhizobium* - *Lespedeza formosa* association.

In a study by Tong and Sadowsky (1994), while copper was observed to uniformly affect growth of *Rhizobium* strains of Tal 634, 640, 380, 1372, 182, 1383, 1820 and 1824 with a Minimum Inhibitory Concentration (MIC) of 80 µg/ml, variations were observed on its effects on growth of *Bradyrhizobium* strains of USDA 61, 110, 122, 123 and 127 with MIC's of 80, 80, 20, 40 and 80 µg/ml, respectively.

Studying the response of rhizobial populations (populations of *Rhizobium leguminosarum* bv. *viciae* nodulating *Vicia sativa* and communities of rhizobial species nodulating *Phaseolus vulgaris*) to moderate copper stress applied to an agricultural soil Laguerre *et al.* (2006) observed that while the genetic structure of *Rhizobium leguminosarum* biovar *viciae* populations was not modified by copper concentrations of up to 91 mg Cu/kgsoil following exposure for up to 3 years, there occurred a shift in the composition of *Phaseolus*-nodulating communities in relation to soil copper content such that three 16S rDNA haplotypes were identified: one corresponding to the *R. leguminosarum* biovar *phaseoli* species, and two others forming a new lineage of *Phaseolus* rhizobia, based on 16S rDNA base sequence analysis. They argued, further, that the reduced frequency of the *R. leguminosarum* species in the *Phaseolus*-nodulating communities from the copper-treated soils was linked to its higher sensitivity to copper as compared to the higher tolerance of isolates belonging to the other rhizobial lineage.

Copper has also been shown to be relatively more toxic to rhizobia *in vitro* than other heavy metals, namely Pb, Hg, Cd, Zn, Cu, and Cr. In a study that compared growth responses of 16 strains of *Bradyrhizobium japonicum*, 15 strains of *Sinorhizobum meliloti*, 24 strains of *Rhizobium leguminosarum (*8 bv. *phaseoli*, 8 bv. *viciae and* 8 bv. *trifolii),* 4 strains of *Rhizobium loti* and 3 strains of *Rhizobium galegale,* Milicic *et al.* (2006) observed that while most strains were found to have relatively higher intrinsic tolerance

to Pb, Zn and Hg, all the tested strains displayed the lowest intrinsic tolerance when growing on Ni and Cu suggesting that Cu, (and Ni) impacted more negatively on the growth of all the tested strains. In that study Milicic *et al*. (2006) observed differences in growth on different concentrations of copper both among different rhizobial species and among strains of the same species, which suggested differences in their genetic structures of tolerance to heavy metal toxicity, probably resulting from their indigenous biodiversity.

2.7.3 Mechanisms of copper toxicity to microorganisms in general

Borkow and Gabbay (2004) postulated that copper becomes toxic to most microorganisms through either (a) alterations in the conformational structures (i.e. damage) of nucleic acids and proteins, (b) cell membrane damage or (c) interference with oxidative phosphorylation and osmotic balance. These three alternative mechanisms may act either singly or in combination to result in damage due to copper toxicity to microbial cells. A few examples in this regard are reviewed in this section.

Rajesh *et al*. (2001) suggested that exposure of *Desulfovibrio desulfuricans* G.20 to Cu(II), Zn(II), and Pb(II) could lead to a decline in membrane integrity and that extensive metal-induced disruption of membrane integrity could be responsible for the observed loss of cell viability, longer lag times, and/or reduced growth of *D. desulfuricans G.20.* Studies based on *Escherichia coli* revealed that copper led to a rapid inactivation of the catalytic clusters of dehydratases - a family of enzymes that has representatives in the central catabolic and biosynthetic pathways (Macomber and Imlay, 2009). They observed that copper rapidly inactivated isopropylmalate dehydratase, an iron-sulfur cluster enzyme in this pathway. A dose of 16 μM copper diminished the total isopropylmalate isomerase (IPMI) activity of cultured cells by 60% within 30 minutes. Similarly, activity decreased in *copA cueO cusCFBA* mutant cells by 80%. In a different study, Baker *et al.* (2010)

found that copper toxicity causes protein damage and oxidative stress through thiol oxidation and the production of oxidative stress responses (OSR) in *Staphylococcus aureus.* They observed that the addition of copper to exponentially growing *S. aureus* resulted in a decrease in the expression of at least 20 genes encoding ribosomal proteins, translation initiation factors, and the RNA polymerase *rpoA* subunit, suggesting that there was a corresponding decrease in protein synthesis upon copper shock. They observed, further, that copper shock also resulted in the induction of the CtsR-regulated misfolded protein response, as addition of copper induced the expression of the *ctsR* repressor gene as well as that of the genes encoding the energy-dependent proteolytic complex *clpC* and *clpP* and encoding the *clpB* chaperone.

2.7.4 Some specific mechanisms of copper toxicity to rhizobia

Though not as widely documented a few reports on mechanisms of copper toxicity specifically to rhizobia are reviewed below. Wayne *et al*. (2002) , for example, observed that a concentration of copper of 2 mM was sufficient to terminate growth of *Rhizobium leguminosarum* WR1-14 and *Sinorhizobium meliloti* RT3-27 at pH of 5.5 and pH 5.7, due to disruption of genes that code for cation-transporting ATPases designated as *actP* (acid-tolerance P-type ATPase), thus preventing the expression of the copper-export system.

Studying the effect of copper toxicity to nitrogen fixation in alfalfa, Porter and Sheridan (1981) reported that Cu^{2+} caused 97 and 100% inhibition of acetylene ($N_2[C_2H_2]$) reducing activity at concentrations of 10 and 100 µg Cu^{2+}/ml, respectively. They found in similar experiments performed with Ranger alfalfa that 100% inhibition of nitrogenase activity occurred at both10 and 100 µg Cu^{2+}/ml. In another study, Bhandal *et al.* (1990) reported

that at 100 ppm, copper (and other heavy metals like Pb, Ni and Cd) caused significant reductions in several nodulation parameters namely nodule numbers per plant, nodule dry weight per plant and number of nodules per gram fresh weight of the root in *vigna radiata*, and this was linked to the inhibition by copper (and other heavy metals) of the acetylene reduction activity of the nodules. The mechanisms of inhibition of acetylene reduction activity are not clearly understood and possibly involve several factors like direct interference of the enzyme protein and reduced availability of the photosynthatate required for the process (Bhandal *et al*., 1990).

2.7.5 Microbial resistance against toxicity of copper: basis for differential performance of different microorganisms

Toxicity to high concentration of heavy metals can be resisted by either (i) diminishing the accumulation of the ion by its active extrusion out of the cell (efflux), (ii) segregating the cation into complex compounds by thiol-containing molecules or (iii) reducing the metal cation to a less toxic oxidation state (Nies, 1999).

According to Dameron and Harrison (1998), the cation translocating P-type ATPases are among the most commonly used mechanisms of resistance by efflux in many organisms. The pump design which is found in a diverse list of organisms and used for a range of metals is believed to be frequently modified to increase specificity and efficacy of the ATPase pumps for a given metal. Copper detoxification in *Enterobacter hirae*, for example, uses a combination of import and export pumps (CopA and CopB respectively) to regulate the intracellular concentration of copper under the control of a copper-regulated operon, an activator CopZ and a represser CopY.

Another commonly used resistance mechanism is the intracellular chelation or sequestration of the metal into harmless complexes. Normally, the chelating agents are either proteins or peptides that eventually form stable complexes with the metal reducing its reactivity and aiding to its excretion out of the cell (Dameron and Harrison, 1998). Yeast cells of *Saccharomyces cerevisiae*, for example, detoxify from excess Cu(I) ions by sequestering them into a tight complex with metallothionein (ScMT). The induction of ScMT by copper is accomplished through a metal-sensing (metalloregulated) transcription factor, ACE1and the later produces a conformational change in ACE1 that increases its affinity for an upstream activation sequence (UAS) in the promoter of the metallothionein gene, leading to increased synthesis of metallothionein (Dameron and Harrison, 1998).

Exclusion by permeability barrier involves the generation of cell surface proteins (collectively called metal detoxification proteins) which bind the heavy metals, producing a barrier that prevents the metals from entering the cell (Meyer, 2001). The bacterium *Escherichia coli*, for example, possess copper ion detoxification systems such as CopA, Cus, and CueO. Santo, *et al*. (2007) found that cells of *E. colli* lacking these systems showed some decrease in their survival rates on copper surfaces. Richards *et al*. (2002) observed blue colonies of *Frankia* strains being formed on the surface of a copper containing medium suggesting that the colonies bound or absorbed the Cu^{2+} in the medium, possibly by producing a diffusible binding compound to the surface of their cells.

Another mechanism is by the cells of microorganisms entering into a VBNC state as indicated in section 2.7.2 above. In this regard Ordax *et al.* (2006) demonstrated that the bacterium *Erwinia amylovora* enters into a VBNC state when exposed to copper toxicity. They argued that the induction of the VBNC state by copper could possibly represent a survival strategy of this bacterium under certain adverse environmental conditions. Copper

is also known to induce *E. coli* to enter the VBNC condition (Brian and Todd, 2001). The fact that high concentrations of copper do not kill all cells exposed to it suggests that current growth-based microbiological methods for assaying toxicity result in an undercount of the number of viable cells through incorrect scoring of VBNC cells as dead (Brian and Todd, 2001).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Soil Characterization and Plant Analysis

3.1.1 Soils and soil characterization

Three soils were used in this study. One was an uncontaminated soil from the Crop Museum – SUA. Another soil was from a coffee farm, and the third soil was taken from a location in a maize field adjacent to the coffee farm in Mbozi, Mbeya Region. The soil from the coffee farm in Mbeya was taken from the Khanji-Lalji farm, whose soils are contaminated due to use of, mainly, blue copper (copper oxychloride). The soil from the adjacent maize farm was similar to that from the coffee farm, but was not contaminated and it served as control soil. The soils were characterised for a few properties known to affect either the symbiotic BNF or the bioavailability of copper added to them, and these properties included pH, Cation Exchange Capacity (CEC), available phosphorus and nitrogen. DiethyleneTriaminePentaAcetic-acid (DTPA) - extractable copper contents of the soils were also determined prior to the MPN study.

The pH of the soil sample was determined by measuring 10 g of soil in a 100 ml broad necked plastic bottle. Using a dispenser 25 ml of water was added and the bottle was closed and shaken in the horizontal position on a reciprocating shaker for 30 minutes at 175 revolutions per minute. The pH of the sample was then read on a digital scale pH meter (previously calibrated using buffer pH 4 and &7 standards).

To determine the CEC, 5 g of an air – dry soil was put in 100 ml Erlenmeyer flask and saturated with 1N NH_4OAc solution and left standing with occasional hand shaking for 24 hours. The saturated soil was then filtered on a Buchner funnel and later washed by 50 ml of NH_4OAc. The soil residue on the Buchner funnel was washed with 50 ml of methyl

alcohol. The filtrate was then distilled using 50 ml of boric acid and the distillate was titrated using 0.1 N H_2SO_4 solution. A blank containing 400 ml of KCl solution only was run for correction purposes and the CEC was calculated as follows:

$$\text{C.E.C. } (Cmol_c(+)/kg \text{ soil}) = \frac{\text{ml of } H_2SO_4 \text{ used} * \text{Normality of } H_2SO_4 * 100}{\text{Weight of the soil sample used}}$$

Available P was determined using Bray 1 method as described by Okalebo *et al*. (1993) where 3.5 g of soil were put in a 100 ml plastic bottle and 25 ml of extraction solution were added. The bottle was tightened and shaken for five minutes. The suspension was filtered at once and 5 ml of the extract was transferred to a 50 ml volumetric flask. 10 ml of a phosphate reagent were added and the mixture was made to the mark by adding distilled water. Twenty minutes later, the absorbance was read on a spectrophotometer at 884 nm wavelength. A standard curve was prepared by plotting absorbance readings of known P concentrations. The absorbance reading of the sample was extrapolated on the standard curve to obtain the P concentration in the sample and the amount of P calculated using the formula:

P concentration in soil ($mgKg^{-1}$) = (X mg * 25*50*1000)/1000 ml *S g*Y

Where: X = Concentration from the graph, S = Weight of soil used, Y = ml of extract pipetted

In the determination of soil available N, a procedure described by Okalebo *et al*. (1993) was followed. One hundred ml of 2 M KCl extraction solution were added to 10 g of freshly sampled soil in a plastic bottle which was carefully stoppered and shaken for 1 hour. The contents were filtered using Whatman No. 42 filter paper and brought to an automatic distillation unit for measurement of available N. Ten ml of the soil extract were added into a 50ml conical flask containing 5 ml of boric acid indicator and 0.2 g of MgO

and 0.2 g of Devarda's alloy and mounted to the distillation unit. The resulting distillate was titrated against 0.002 N H_2SO_4 and the available N was calculated as follows:

$$\text{Available N} = \frac{\text{ml of 0.002 N } H_2SO_4 \text{ used} * 28\mu g}{\text{Weight of soil used}}$$

To determine DTPA - extractable copper, 20 g of soil were transferred to a plastic bottle and 40 ml of buffered DTPA extraction solution were added. The mixture was shaken for two hours and the suspension was filtered immediately after shaking into a 100 ml plastic bottle. The copper content of the extract was measured using an Atomic Absorption Spectrophotometer (AAS) machine which gave absorbance readings of the sample. A standard curve was prepared using absorbance readings of known Cu concentrations. To get the concentration of Cu in the sample, the absorbance reading of the sample was extrapolated on the standard curve and calculated as follows:

$$\text{Concentration of Cu in soil (mg/Kg)} = \frac{C * \text{ml of DTPA used} * 1000\ g}{1000 * \text{Weight of soil used}}$$

Where: C = concentration Cu from the graph

3.1.2 Properties of the soils used

The characteristics of the soil used in this study are summarised in Table 1.

The pH of both soils from Mbozi and SUA ranged from 6.5 to 6.6 hence slightly acidic. These pH values needed no adjustments as they were in the range that can support BNF as all the root nodule forming bacteria prefer near neutral reactions of the growth medium (Vincent, 1970).

The C.E.C. values of all the soils ranged from 14.9 to 17.1 Cmol(+)/kg soil. These values were within the medium ranges according to Msanya *et al.* (2001) reflecting a moderate capacity of these soils to adsorb cations (Cu^{2+} inclusive) on its colloidal surfaces.

Similarly, Bray I P values of the used soils ranged from 5.1 to 7.8 mg kg^{-1} which based on a report by Msanya *et al*. (2001) are on their lower side. Thus, the fertility status of the soils with respect to Phosphorus was poor which would have impacted the symbiotic NBF negatively. However, the soil from SUA, Morogoro which was used for the pot experiment was supplemented with fertilizer P in the form of Triple Super Phosphate (TSP) to attain an equivalent rate of 40 kgP/ha.

Available N values were between 13.8 and 17%. These values were low with respect to N fertility of the soils generally. The contaminated soil from Mbozi, indeed, had a high level of (DTPA-extractable) copper as compared to the other soils (Table 1), and could be expected to affect rhizobial growth.

Table 1: Characteristics of the soil used

Parameter determined	Soil from SUA	Soil from Mbozi, Mbeya	
		CN*	CS*
CEC (Cmol(+)/Kg	17.1	15.5	14.9
pH (in water)	6.6	6.5	6.5
Available N (µg/kg soil)	17.0	15.7	13.8
Bray-1 P (mg/k gsoil)	6.2	7.8	5.1
DTPA- extractable Cu (ppm)	0.9	82.5	1.8

CN* = Contaminated soil, CS* = Control (uncontaminated) soil.

3.1.3 Plant analysis

Plant analysis was done with respect to the determination of total plant nitrogen. 0.2 g of a plant material was put into a digestion tube in which a mixed catalyst (K_2SO_4 $CuSO_4$ and Selenium powder in ratios of 10: 1: 0.1) were added. Twenty ml of concentrated sulphuric acid were also added to the mixture which was then digested on a hot plate at 360ºC for

two hours. Five ml of a cooled digest of plant material was mixed with 10 ml of 40% NaOH and distilled into 5ml of 1% boric acid containing 4 drops of a mixed indicator. The distillate was titrated with 0.002 N H_2SO_4 solution and corrected using a blank reading. Percentage of nitrogen in the plant sample was calculated as follows:-

% N = ((ml of 0.002 N H_2SO_4 used –blank reading) * 0.2)/weight of sample

3.2 Determination of Copper Levels that Caused Lethal Effects to Rhizobial Cells *in vitro* and *in vivo*

Two strains of bradyrhizobia (CP_1 for cowpea and GM_8 for soybeans) and two of rhizobia (PV_1 and PV_2 for field beans) were used in testing their ability to proliferate *in vitro* under increasing copper levels and ability to survive *in vivo*.

The four strains (PV_1, PV_2, CP_1 and GM_8) were previously isolated and maintained at 4ºC on slants of Yeast-extract Mannitol Agar (YMA) in the Department of Soil Science-SUA, following the procedure described by Vincent (1970). One ml suspensions of a pure culture of each of the four strains was introduced in a liquid culture medium (Yeast-Extract Manitol Broth) treated with predetermined amounts of the stock solution of 1000 ppm of $CuSO_4$ to achieve final concentrations of 0, 20, 40, 60, 80 and 100 ppm of copper to cover the range of concentrations that are commonly found in agricultural soils, as shown in Appendix 1. The treatment(s) were replicated three times and incubated on an automatic shaking bath for seven days. At the end of the seven - day incubation period, the number of bacterial cells (CFU /ml) were determined by the Miles and Misra drop-plate procedure as described by Vincent (1970) to reflect the effects (if any) of increasing copper levels on the strains' proliferating ability in the liquid medium.

In a parallel sub-set, 1ml of a pure culture of each of the four strains was introduced in 10 g of previously sterilized soil treated with predetermined amounts of the stock solution of 1000 ppm of $CuSO_4$ to achieve final concentrations of 0, 40, 80 and 100 ppm Cu as shown in Appendix 2. The treatments were replicated 3 times and incubated for 0, 2, 4, 6 and 8 weeks. At the end of the 0, 2, 4, 6 or 8 week- incubation period, the population sizes of the bacteria (CFU/gsoil) were determined respectively by soil dilution plating to check for the ability of each of the strains to survive in a copper-treated soil, as this may be different from proliferation ability of the same in a culture medium.

3.3 Determination of Inhibitory Levels of Copper on Nodulation and Symbiotic BNF *in vitro* and *in vivo*

The effect of increasing copper levels on the ability of each of the four strains (PV_1, PV_2, CP_1 and GM_8) to nodulate their respective host legumes and fix nitrogen was examined in Leonard jars and in native (non-sterile) soil. One ml 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, as shown in Appendix 3. The 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.

At the end of the five - week period the plants were harvested and three parameters were determined in the laboratory: (a) nodule fresh weight, (b) nodule fresh volume and (c) total plant (shoot) nitrogen. A sensitive electronic balance was used to measure nodule fresh weights of the harvested plants. To measure the nodule fresh volumes, a measuring cylinder carefully cut on both ends and sealed by parafilm paper on one end was used

(Msumali and Kipe – Nolt, 2002). Total nitrogen was determined using the Kjedahl wet digestion procedure as described by Anderson and Ingram (1993).

In a parallel pot experiment, soil from the SUA Crop Museum was used. The value of 17% N for the soil from SUA, Morogoro which was used for the pot experiment was considered to be too high for this research as it would have served as a cheaper source of N (Evers, 2002) and, therefore, would interfere with the symbiotic BNF process for which this study was based. Based on the results of the laboratory characterization of the soil, the relatively high level of available N was lowered far down from the original of 17 to 0.8µgN/gsoil. This was done through treatment of the soil with maize bran at an equivalent rate of 5 tons per hectare and incubated for six weeks to allow the microbial community to immobilize the available N in the soil given a wide C: N ratio carbon source.

This potted soil was then treated with predetermined amounts of $CuSO_4$ to achieve final concentrations of 0, 20, 40, 60, 80 and 100 µg Cu/g soil, as indicated in Appendix 4. Four seeds were planted per pot inoculated with 1ml 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 for 35 days for the bacteria to nodulate their host legume and fix nitrogen. At the end of the five - week period, the plants were harvested and the three variables namely: nodule fresh weight, nodule fresh volume and total plant nitrogen were determined in the laboratory by procedure described above.

3.4 Comparison of Rhizobial and Bradyrhizobial Populations in Cu-contaminated Soils using the MPN Plant Infection Technique

Top soil (0 -15 cm) was sampled from the Kanji-Lalji coffee farm in Mbozi Tanzania. The farm has been under constant copper–based fungicides application for over the past 30 years (Halid, H.I., personal communication). The most frequently copper-based fungicides used in control of coffee diseases such as the Coffee Berry Disease (CBD) at the Kanji-Lalji farm over the years is Blue copper (Copper Oxychloride Wettable powder). An adjacent non-copper treated field cropped to maize was used as a control. In each of the two soils, DTPA -extractable copper was determined in the laboratory following a procedure as described by Anderson and Ingram (1993). The big difference between the control and the experimental fields in terms of their content of DTPA-extractable copper (1.8 versus 82.5 ppmCu) warranted a good case on which this study was based. So rhizobial and bradyrhizobial populations in each of the two soils were determined using the Most Probable Number (MPN) plant infection technique as described by Anderson and Ingram (1993). The experiment was considered to be a 2 x 4 factorial from which ANOVA and subsequent mean separation were performed.

3.5 Statistical Analysis

The entire study was considered to be a two - factor factorial experiment organised in a Completely Randomised Block Design (CRBD). The experiments, therefore, (except the MPN which was a 2 x 4 factorial) were 6 x 4 factorial, replicated three times, in which the first factor was copper concentration (with six levels) and the second factor was rhizobia strain type (with four levels). Statistical analyses were done using both excel and Statistical Analysis Systems softwares (SAS Institute, 2007). A general model used for the analysis was, therefore,

$Y_{ij} = \mu + C_i + S_j + CS_{ij} + e_{ij}$

Where: μ = Overall mean

C_i = effect of the i^{th} treatment C (Copper level)

Sj = effect of the j^{th} treatment S (Strain type)

CSij = interaction effect between treatments C and S

eij = residual error term

Subsequent separation of copper treatment means was done according to the New Duncan's Multiple Range (DMR) test, at the 0.05 probability level.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1. Effects of Cu on Proliferation of Rhizobia *in vitro*

Table 2 shows the effect of Cu on rhizobial proliferation. The results indicated that there was a progressive decrease in the number of the symbiotic nitrogen fixing bacteria with successive increase (0 to 100 ppm) in the concentration of copper in the liquid medium. This was more pronounced for the slow – growing bradyrhizobial strains (CP_1 and GM_8) than for the fast – growing rhizobial strains (PV_1 and PV_2).

Analysis of Variance (ANOVA) (Appendix 5) indicated, overall, that there were significant ($p = 0.05$) differences in response to copper toxicity among the four strains (the basic ANOVA table itself). It was observed, further, that while strains of the slow - growing bradyrhizobia (CP_1 and GM_8) exhibited no growth at all at copper concentrations of 80 ppm and above (Table 2), substantial growth of the fast - growing rhizobia (PV_1 and PV_2) occurred at these concentrations. Furthermore, it was observed that the overall proliferation abilities among the slow – growing bradyrhizobial strains CP_1 and GM_8 in the copper amended medium were not significantly ($p = 0.05$) different at the t-grouping level (Appendix 5). However, in the same t-grouping comparison, the fast – growing strains exhibited significantly ($p = 0.05$) different abilities to proliferate in the copper amended medium. The means appearing in Table 2 can be explained as follows. The overall ranking of all means is presented at the end of Appendix 5, ranked according to Duncan's New Multiple Range Test.

Table 2: Effects of copper on proliferation of rhizobia/bradyrhizobia *in vitro*

Copper concentration (ppm)	Mean rhizobial population sizes (Log_{10} CFU*/ml)			
	Fast – growers		Slow – Growers	
	PV_1	PV_2	CP_1	GM_8
0	10.010abc	10.167a	9.893bc	9.880bc
20	9.979abc	10.115ab	9.822cd	9.826cd
40	9.053f	9.301e	8.418gh	8.519g
60	9.204ef	9.587d	8.000i	8.201hi
80	8.667g	9.038f	0.000j	0.000j
100	8.519g	8.560g	0.000j	0.000j

Means in the same column followed by the same letters are not significantly different (P = 0.05) based on the New Duncan's Multiple Range (DMR) Test.

*CFU = Colony Forming Units.

The means appearing in Table 2 are a result of rearrangement of the Duncan-ranked means according to strain type and copper concentration level. Hence, these means are different from the overall means of strains as grouped by the't-grouping'. The means in Table 2 show, therefore, the strain x copper level interaction. This concept was followed for the subsequent Tables (Tables 3-7), and these appear in relevant appendices.

The observed decrease in the number of bacteria with successive increases in the concentration of copper can be attributed to the fact that increasing Cu concentration resulted in an increase in the intensity of Cu toxicity to the rhizobia, hence more and more cells became susceptible to toxicity effects of copper at higher concentrations.

Toxicity effects of copper to growth of rhizobia *in vitro* have also been reported by other workers. At concentrations as low as 10 ppm copper has been reported to impair growth of *Rhizobium leguminosarum* (Alexander *et al*., 1999; Milicic *et al*., 2006), *Bradyrhizobium*

japonicum, *Sinorhizobium meliloti*, *Rhizobium loti* and *Rhizobium galegae* (Milicic *et al*., 2006). It is necessary to state that in the present study, impaired growth only became apparent at 40 ppm Cu and above.

Variations in response to copper toxicity *in vitro* among strains of rhizobia (ANOVA table in Appendix 5) and between the fast – and slow – growing strains observed in this study (Table 2) could be a result of inherent differences in the ability to tolerate copper toxicity stress linked to their genetic biodiversity. It has been reported in other studies that variations in response to copper toxicity existed not only between the fast - and slow - growing rhizobia (Tong and Sadowsky, 1994) but also among different species of rhizobia and even among strains of the same species (Milicic *et al*., 2006).

Although definite conclusions cannot be made based on only the four strains used in this study, the observation that the slow – growing bradyrhizobial strains of CP_1 and GM_8 behaved more similarly in response to copper toxicity while the fast – growering PV_1 and PV_2 showed significantly ($p = 0.05$) different responses to copper toxicity stress could possibly be due to greater biodiversity in the fast – growing rhizobia than within the slow –growing rhizobia, as the former is a relatively larger group than the latter (Giller and Wilson, 1991). Hence, there wa less uniformity in behaviour of the members of the former group. This would suggest, further, that the mechanisms of resistance to copper toxicity are probably more evolved and more efficient in the more diverse group (rhizobia) as compared to the less diverse one (bradyrhizobia). This would also imply, conversely, that mechanisms of susceptibility are more pronounced in the relatively smaller group, the slow – growers.

Observations in previous studies by Milicic *et al.* (2006), indicating presence of differences in the ability to tolerate copper (and other heavy metals') toxicity, not only among different groups or species of rhizobia but also among strains of the same species of rhizobia/bradyrhizobia, suggest, therefore, as noted above, that there exist differences in the genetic characteristics responsible for susceptibility or tolerance to heavy metal toxicity both between species of rhizobia and also among strains of the same species, and this is linked to their genetic biodiversity.

One of the strategies of some microorganisms (rhizobia inclusive) to resist copper stress is by cells entering into a Viable But Non-Culturable (VBNC) state (Ordax *et al.*, 2006; Brian and Todd, 2001). Other resistance mechanisms, as detailed under section 2.7.5 of this dissertation, could include intracellular chelation or sequestration of the copper into harmless complexes (Dameron and Harrison, 1998), production of exopolymers (extracellular polymeric substances) that bind metallic cations to form immobilized complexes that have reduced toxicity (Watcharamusik *et al*., 2008), and development of a permeability barrier made up of cell surface proteins (collectively called metal detoxification proteins) which bind the heavy metals, producing a barrier that prevents the metals from entering into the cell (Meyer, 2001). Thus, any of these mechanisms of resistance could possibly be more pronounced in the fast – growing rhizobia, making them more resistant to the Cu toxicity and hence be able to proliferate more as compared to the slow – growing bradyrhizobia.

4.2 Effects of Cu on Survival of Rhizobia/bradyrhizobia *in vivo*

Results of this experiment (Fig. 1, Appendix 6) indicated that there was, overall, a general reduction in the population size of rhizobia with increasing copper levels, but the reduction was more pronounced in the slow – growing group of the root nodule bacteria.

This trend is also generally apparent, especially at the higher levels of copper (compared to the controls), when individual strains are examined (Fig. 2).

ANOVA (Appendix 6) showed that there were significant (p = 0.05) differences in survival to copper toxicity stress among the four strains. Although the step-wise increase in Cu concentration caused significant (p = 0.05) reductions in the numbers of rhizobia and/or bradyrhizobia in all the four strains, only slight differences tended to exist on the survival patterns of the fast – and slow – growing rhizobia (Fig. 1).

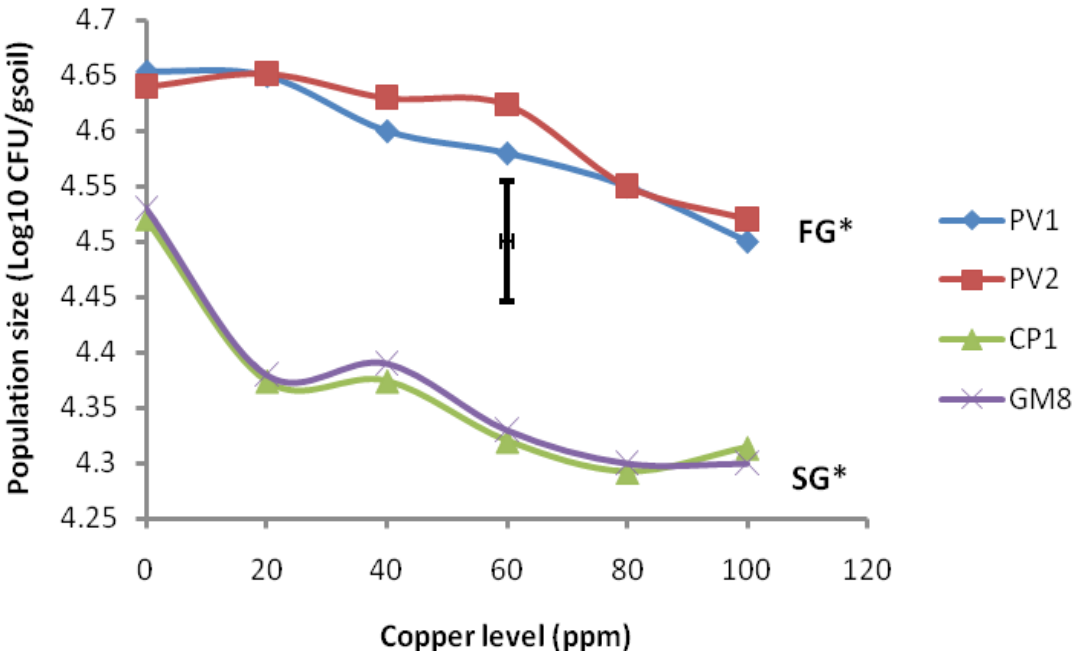

Figure 1: Overall effect of copper on survival of rhizobial and bradyrhizobial strains *in vivo* averaged over sampling times

Bar indicates 0.05 level of probability.

FG* = Fast – growing rhizobia,

SG* = Slow – growing rhizobia

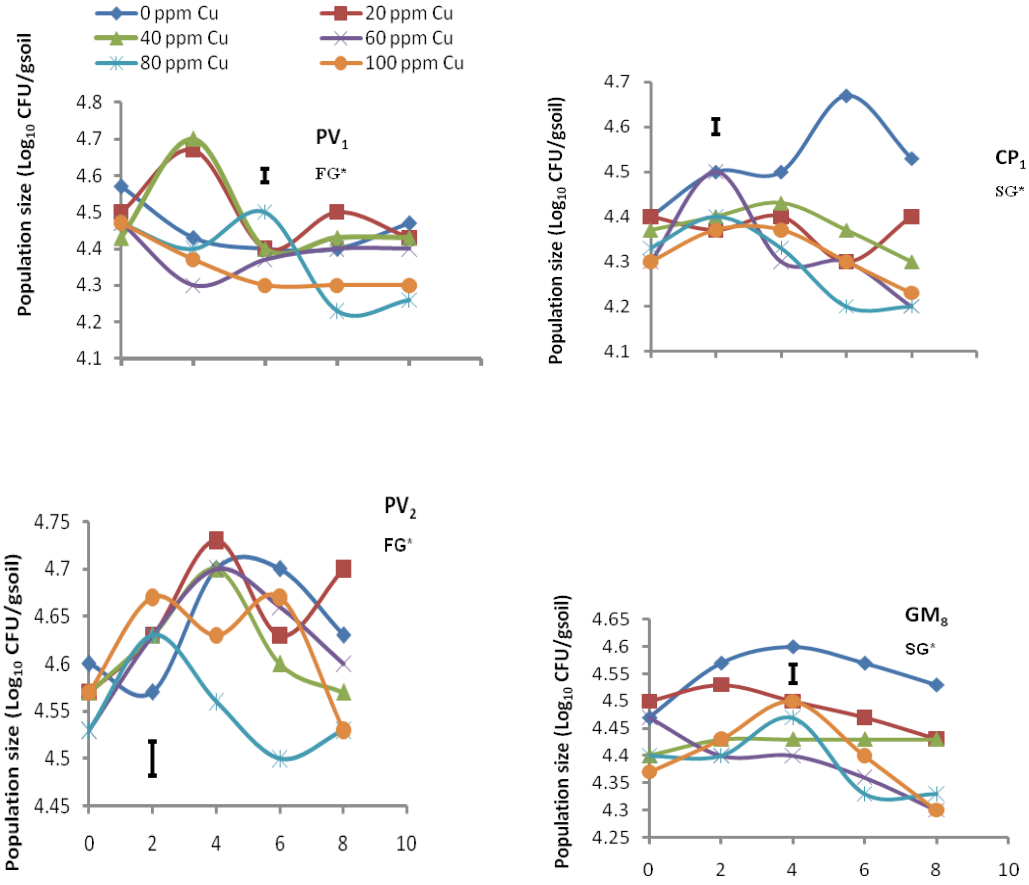

Figure 2: Effects of Cu levels on survival of individual rhizobial strains *in vivo* (across sampling times)

Bars indicate 0.05 level of probability; FG* = Fast – grower; SG* = Slow – grower

On the whole, comparing the population differences (calculated from the 0 and 100 ppm Cu treatments) i.e. 0.16 and 0.12 Log_{10} CFU/gsoil for PV_1 and PV_2, and 0.20 and 0.23 Log_{10} CFU/gsoil for CP_1 and GM_8, respectively, would seem to indicate, as noted above, a greater tolerance of the fast – growing rhizobial strains than the slow – growing bradyrhizobial strains to copper toxicity, a finding which is consistent with the *in – vitro* study reported above (Table 2).

A further observation is the fact that the toxicity effects were more pronounced in the solution culture than in the soil (c.f. Table 2 and Fig. 1). Table 2 shows that at copper concentration equal to, and beyond, 80 ppm, the proliferation of slow- growers *in vitro* was completely inhibited. This complete inhibition in the growth of the strains was not evident in soil at comparable levels of copper (Fig. 1). It was observed that even at 100 ppm Cu, the slow - growers maintained fairly high populations in soil (i.e. 4.30 log_{10} CFU/gsoil).

The survival picture of each of the four strains was evaluated when the time factor (i.e. incubation period) was examined, at different levels of copper (Fig. 3). It was observed that at each level of copper, the populations of rhizobia were different between the strains, even if the overall trends were not always identical (Fig. 3). These differences were statistically ($p = 0.05$) different (Appendix 6). There was also a tendency of the populations increasing, sometimes up to the fourth week of incubation, before an overall decrease was observed subsequently (Figs. 2 and 3).

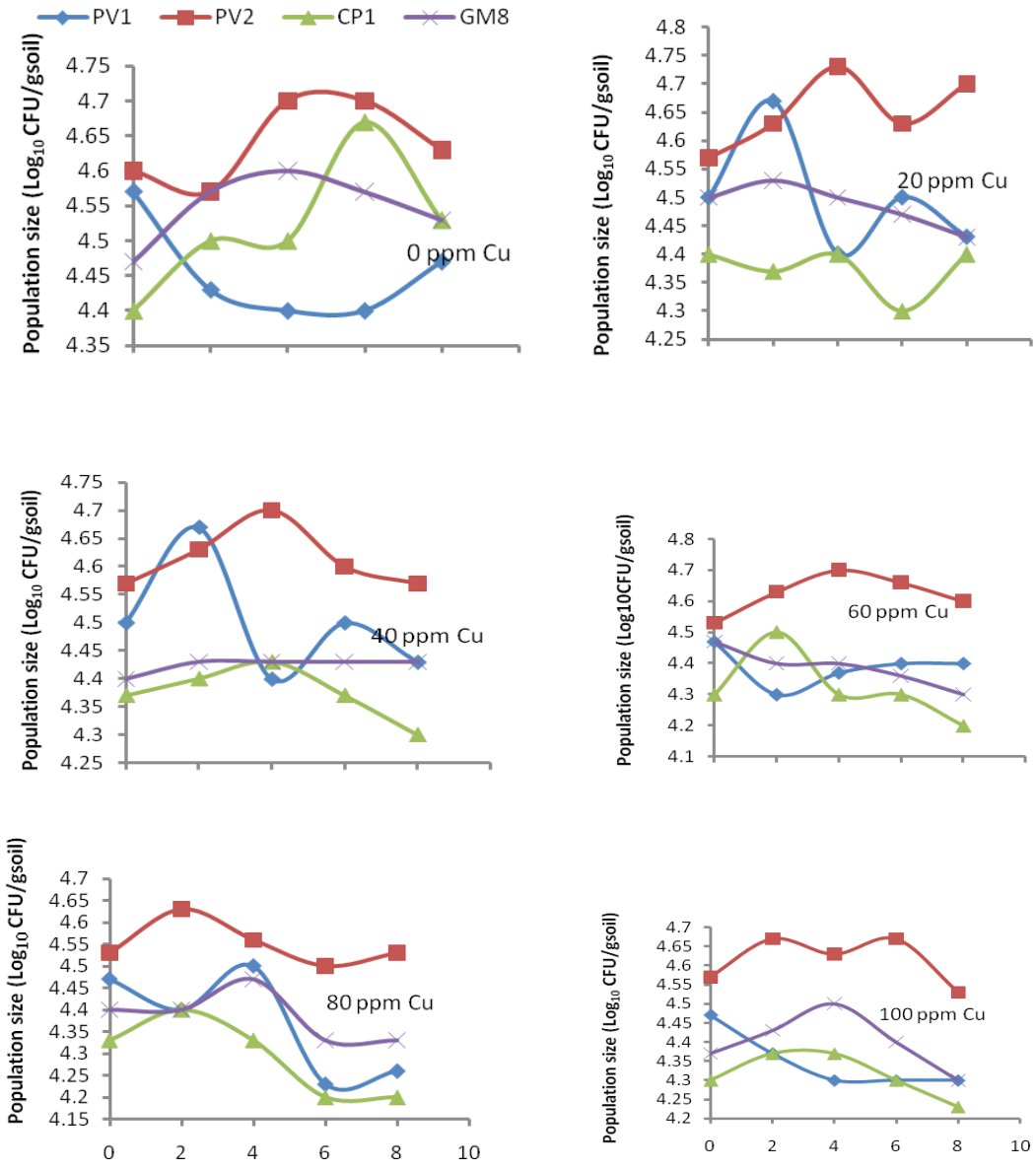

Figure 3: Effect of sampling time on survival of rhizobial strains at different copper levels *in vivo*

The observed general reduction in the population sizes of rhizobia/bradyrhizobia with increasing copper levels both for the fast - and slow - growing groups of the root nodule bacteria implied, as noted before (section 4.1), that increasing the concentration of Cu in soil resulted in a general increase in the intensity of Cu toxicity to rhizobial strains and, thus, to greater susceptibility of rhizobial cells in soil.

Variations in the survival abilities among strains and between the fast - and slow - growers observed in this study would imply, as pointed out in section 4.1 above, that there were differences in abilities to resist copper toxicity stress among the strains, possibly due to differences in their inherent biodiversity.

The relatively lower intensity of copper toxicity at higher concentrations in soil - based studies thereby maintaining relatively higher populations as compared to solution culture, as observed above, imply that the soil medium had a sparing influence on the toxicity effect of copper to the microorganisms. This can be attributed to strong chelation properties of soil organic colloids, causing the ionic form of copper (i.e. Cu^{2+}) to be held more tenaciously by the soil colloids (Schulte and Kelling, 1999), thereby reducing its bioavailability and its toxicity to rhizobia in the soil. It is important to note, further, that very often, the response of microorganisms to antimicrobial substances when evaluated *in vitro* does not necessarily relate to the reaction of the same *in vivo* (Diatloff, 1970). In this regard, Msumali and Ng'ang'a (2007) reported that two types of insecticides, *Lannate* (a carbamate) and *Karate* (a pyrethroid) each inhibited the growth of heterotrophic bacteria, fungi and actinomycetes *in vitro* although the same insecticides did not affect the growth of microorganisms *in vivo* in soil, neither did they affect important processes such as the decomposition of green manure in the soil.

The observation that the mean population sizes of all the four strains increased during the first four weeks of exposure in the different levels of copper, indicated that growth of the four strains was not initially suppressed by copper within a relatively shorter period of exposure, especially at the lower copper levels, although prolonged exposure to eight weeks reduced their numbers significantly ($p = 0.05$) at all copper concentrations. The initial increase in populations may be related to initial binding/complexing of the copper by soil's organic matter. The subsequent decrease in populations may be due to subsequent release of the Cu from the organic matter, possibly due to its decomposition beyond four weeks of incubation.

4.3 Effects of Copper on Nodulation and Symbiotic Biological Nitrogen Fixation

4.3.1 Effect of elevated levels of copper on nodulation

4.3.1.1 Nodule volumes

Table 3 shows the results of the effect of copper on fresh nodule volume *in vitro*. Increasing the concentration of copper resulted in a significant ($p = 0.05$) 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 *in vitro*. The decrements were, however, more pronounced only for the two strains of the slow – growing bradyrhizobia than for the fast – growing rhizobia (c. effective decrements of 18.1 and 5.6% for PV_1 and PV_2 respectively, against 88.6 and 90.3% for CP_1 and GM_8, respectively, as the 0 and 100 ppm Cu levels are compared (Table 3). ANOVA (Appendix 7a) indicated that there were significant ($p = 0.05$) differences in response to copper toxicity stress among the four strains.

Table 3: Effects of copper on nodule volume *in vitro*

Copper concentration (ppm)	Nodule volumes (ml/plant) under			
	Fast–growing rhizobia		Slow–growing rhizobia	
	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

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.

A similar pattern of response was observed in vivo in that increasing the concentration of copper in potted soil resulted in significant (p = 0.05) reductions in fresh nodule volumes for all the four strains of PV_1, PV_2, CP_1 and GM_8. Results (Table 4) showed that although the decrements were generally progressive across all the four strain in vivo there were substantial differences between the two groups, with larger decrement values (when the 0 and 100 ppm Cu levels were compared) of 37.3 and 39.2% for the slow – growing GM_8 and CP_1 strains against 17.2 and 18.2% for the fast – growing PV_1 and PV_2 strains.

The fast – growing strain PV_1 and PV_2 showed significant (p = 0.05) differences in their mean fresh nodule volumes in vivo (Appendix 7b). However, the mean fresh nodule volumes of the slow – growing bradyrhizobial strains of CP_1 and GM_8 were not significantly (p = 0.05) different.

Table 4: Effects of copper on nodule volume *in vivo*

Copper concentration (ppm)	Nodule volumes (ml/plant) under			
	Fast – growing rhizobia		Slow – growing rhizobia	
	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

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.

4.3.1.2 Nodule masses

Copper did also affect nodule mass in a fashion similar to its effect on nodule volume. Increasing copper concentration caused a gradual but significant (p = 0.05) decrease in fresh nodule mass in all the four strains under the *in vitro* study (Table 5, Appendix 8a). However, the effects *in vitro* were again more pronounced in the slow – growers, with effective decrements of 94.2 and 83.7% for CP_1 and GM_8, respectively (when the 0 and 100 ppm Cu levels were compared), against only 50.3 and 50.7% for the fast- growing PV_1 and PV_2, respectively (Table 5). Unlike with the fresh nodule volumes as presented above, the fast – growing rhizobia, PV_1 and PV_2 showed no significant (p = 0.05) differences in their mean fresh nodule masses. However, significant (p = 0.05) differences were observed between the two slow – growing bradyrhizobial strains, CP_1 and GM_8.

Table 6 shows results of the effects of copper on fresh nodule mass *in vivo*. Similarly, increasing copper concentration resulted in significant (p = 0.05) decreases in fresh nodule mass *in vivo* for all the four strains. However, the effects of copper on fresh nodule mass

Table 5: Effect of copper on nodule mass *in vitro*

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

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.

Table 6: Effects of copper on nodule mass *in vivo*

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 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.

in vivo were of a relatively larger negative impact on bradyrhizobial strains (effective decrements of 71.4 and 60.8% for CP_1 and GM_8, respectively) than on rhizobial strains PV_1 and PV_2 (effective decrements of 19.1 and 12.2%, respectively), as the 0 and 100 ppm Cu levels were compared. However, the mean fresh nodule mass of strain GM_8, a slow – growing strain, was not significantly ($p = 0.05$) different from that of PV_2, a fast – growing strain (Appendix 8b).

The observation that increasing the concentration of copper resulted in impaired nodulation as reflected by reduced fresh nodule volumes and masses both *in vitro* and *in -vivo* could again be explained by the fact that the intensity of Cu toxicity increased as the concentration of copper was increased, hence each successive increase in Cu concentration causing more and more harm to the respective nodulation. The results of this study are in total agreement with reports of other workers in this regard that elevated levels of copper in a growth medium can impair nodulation of legumes by rhizobia. Younis (2007), for example, linked the impaired nodulation in rhizobia – legume symbioses to the ability of copper to interfere with protein synthesis, resulting in severe reductions in protein content of both nodule cytosol and bacteroid fractions and reduced leghaemoglobin concentration of nodule cytosol.

The general observation that the fast – and slow – growers behaved differently, with copper exerting greater negative impacts to the symbioses of slow – growers than to those of the fast – growers with respect to nodulation under copper toxicity stress alludes to the contention made earlier that the mechanisms of resistance (or tolerance) to copper toxicity stress may be more efficient in one group (the fast – growers) than the other (the slow– growers), therefore, making the slow – growers, and the symbioses they are in, more vulnerable to copper toxicity stress.

It is important to note, however, that the observed impairment of nodulation in established rhizobial – legume associations may not be an exclusive effect of copper on the microsymbiont(s) alone. Instead, copper may also exert its negative influences on the macrosymbiont(s) as well, hence aggravating the impairment of the nodulation process. Elevated levels of copper in the growth medium have been reported to result in severe deformations of root cuticle, with symptoms ranging from reduced root growth to disruption of root hair formation (Menzies, 2004). Similarly, Baijukya (1996) reported, in this respect, inhibition of nodulation by Kocide 101 (77% cupric hydroxide) when applied at two to four times the recommended rates, mainly due to the direct effect of the copper fungicide on the bean plant.

4.3.2 Effect of copper on the process of symbiotic biological nitrogen fixation

4.3.2.1 In vitro studies

Results of the effect of copper on total shoot nitrogen *in vitro* are presented in Table 7. There was a general decrease in total shoot nitrogen as the concentration of copper was increased from 0 to 100 ppm. It was observed that the fast – growing rhizobia suffered a far less impact with shoot total nitrogen reductions (when the 0 and 100 ppm Cu levels were compared) of only 10.4 and 10.5% for strains PV_1 and PV_2, against 28.8 and 56.9% for the slow - growing bradyrhizobial strains of GM_8 and CP_1, respectively (Table 7). ANOVA (Appendix 9a) showed that there were significant ($p = 0.05$) differences among the four strains and that the fast - growers behaved more similarly with no significant ($p = 0.05$) differences in their mean shoot total nitrogen contents *in vitro*. However, the slow – growing CP_1 and GM_8 had significantly ($p = 0.05$) different mean shoot total nitrogen contents.

Table 7: Effect of copper on plant total nitrogen *in vitro*

Copper concentration (ppm)	Shoot total N (%) in legumes due to:			
	Fast–growing rhizobia		Slow-growing rhizobia	
	PV_1	PV_2	CP_1	GM_8
0	1.64hi	1.62ij	3.23a	2.57b
20	1.63i	1.61ij	2.59b	2.28c
40	1.55ijkl	1.55ijkl	1.74gh	2.14d
60	1.56ijkl	1.53ikjl	1.61ij	2.03e
80	1.53ijkl	1.50bjklm	1.58ijk	1.91f
100	1.47klm	1.45lm	1.39m	1.83fg

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 Test.

4.3.2.2 In vivo studies

Table 8 shows the results of the effect of copper on shoot total nitrogen *in vivo*. Consistently, there was a general decrease in shoot total nitrogen as copper was increased from 0 to 100 ppm. It was observed further that the reductions in the values of total nitrogen per plant *in vivo* were higher for the slow - growing bradyrhizobial (c. 39.2 & 36.2% for GM_8 and CP_1) as compared to the fast - growing rhizobia (c. 17.2 & 18.2% for PV_1 and PV_2 respectively), as the 0 and 100 ppm Cu were compared.

Similar to the observations for rhizobial proliferation (section 4.1), survival *in vivo* (section 4.2) and fresh nodule volume (section 4.3.1.1) above, there were clear differences in the pattern of response to copper toxicity stress between the fast – and slow – growers, with slow – growers consistently showing no significant (p = 0.05) differences in their mean shoot total nitrogen *in vivo*. However, like the observations made earlier in this respect, the fast – growers showed significantly (p = 0.05) different mean shoot total nitrogen contents *in vivo* (Appendix 9b).

Table 8: Effect of copper on plant total nitrogen *in vivo*

Copper concentration (ppm)	Shoot total N (%) in legumes due to:			
	Fast–growing rhizobia		Slow-growers rhizobia	
	PV_1	PV_2	CP_1	GM_8
0	4.95a	4.71abcd	4.85ab	4.98a
20	4.59bcde	4.41defg	4.74abcd	4.76abc
40	4.60bcde	4.44cdef	4.73abcd	4.79ab
60	4.59bcde	4.25fg	3.77i	3.76i
80	4.28efg	4.13fgh	3.45j	3.28jk
100	4.10gh	3.87hi	3.09k	3.03k

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.

The general reduction in shoots total nitrogen as copper was increased from 0 to 100 ppm observed above is a reflection that at higher concentrations copper inhibited symbiotic biological nitrogen fixation as well, in addition to inhibiting rhizobial proliferation and nodulation as already presented. This general inhibition was due to the fact, as noted previously, that increasing the concentration of copper resulted in a corresponding increase in the intensity of toxicity of copper to the symbiotic BNF machinery, hence reducing its efficiency to fix N_2. The increased inhibition of nitrogen fixation at higher Cu levels has also been reported by other workers.

Porter and Sheridan (1981), for example, reported that 100 µg Cu^{2+}/ml caused a 100% inhibition of acetylene ($N_2[C_2H_2]$) reducing activity in alfalfa *in vitro*, and also that nitrogenase activity was completely inhibited by the same concentration of copper. Barik and Chandel (2002) reported from a field experiment that Cu at 10 kg/ha significantly reduced the nodule leghaemoglobin content in four cultivars of soybean, namely Bragg, PK 416, PK 262 and PK 1042 inoculated with *Bradyrhizobium japonicum.*

The observation that the two fast – growing strains of PV_1 and PV_2 had significantly ($p = 0.05$) different mean total shoot nitrogen contents and that the slow – growing strains of CP_1 and GM_8 did not result in significantly ($p = 0.05$) different total N contents was, as noted above, consistent with results under fresh nodule volume *in vitro*, fresh nodule volume *in vivo* and rhizobial proliferation studies reported previously in this dissertation. This observation, though not as clearly maintained under fresh nodule mass studies, could be a reflection of the fact that the fast – growers belong to a relatively larger and more evolved group of root nodule bacteria and, therefore, have greater genetic biodiversity within this group, than in the relatively smaller group of slow – growing bradyrhizobia.

4.4 Occurrence of Rhizobial and Bradyrhizobial Strains in Soils with Elevated Levels of Cu in the Field

Results of this study are presented in Table 9. ANOVA showed that there were no significant ($p = 0.05$) differences in population sizes between the uncontaminated control soil (with 1.8 ppm Cu) and contaminated soil (with 82.5 ppm Cu) for each of the four indigenous rhizobial strains studied. There were, however, generally lower populations (though not statistically different) of the slow – growers in the contaminated soil.

The observed general tendency of copper toxicity depressing more the slow – growers than the fast – growers is consistent with previous results (section 4.1 to 4.4). The lack of a significant effect of this depression may be due to adsorption of copper in the contaminated soil, thereby reducing the effective concentration of copper in the soil solution of the contaminated soil. The relatively greater tolerance to copper in the case of the fast-growers may also be related to long term adaptation of these rhizobia to higher total copper levels in the contaminated soils.

Table 9: Most Probable Number of rhizobial populations in copper contaminated and uncontaminated soils

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

Means 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.

Bearing in mind that these rhizobia reported under Table 9 and Appendix 10 may be entirely different from those strains studied under *in vitro* and *in vivo* conditions, i.e. strains PV_1 and PV_2 (for common bean) and strains CP_1 and GM_8 for cowpea and soybean, respectively, (sections 4.1 to 4.4), there was a general consistency in the relative responses to copper toxicity between different levels of copper contamination. This, however, may be unequivocally proven by including more strains of the two respective categories of rhizobia in future studies, for more reliable conclusions to be drawn.

The toxicity effects of copper to rhizobia as discussed herein are wide, and may range from reducing growth and survival of the free living root nodule bacteria cells to inhibitory effects on nodulation and N_2 fixation by established rhizobia/bradyrhizobia – legume associations. This key observation is well reflected by studies of other workers as pointed out under literature review of this dissertation. Copper was shown to affect both growth of free living rhizobia (Alexander *et al*., 1999) and the performance of the rhizobial/bradyrhizobial – legume associations (Nie *et al*., 2002; Younis, 2007), as has also been amply shown in the present studies.

In the present study, however, the response to copper toxicity involved only four strains. These four are too few to allow definite conclusions to be drawn. Future studies should involve more strains so that the relative responses, in particular, between the slow – and fast – growers can be assessed if real differences indeed exist.

Although an insight into mechanisms of copper toxicity (or resistance) was not part of the present study, and, could not be ascertained based on the few (four) strains studied, it is important to note that Giller and Wilson (1991) have outlined what they regarded as *key differences* between rhizobial genera, namely between *Rhizobium*, *Bradyrhizobium* and *Azorhizobium*. Those differences were based mainly on cell morphology/anatomy, DNA-base contents, carbohydrate metabolism and N_2 fixation environments. Therefore, the basis of the differences in response (or tolerance) to copper toxicity in the strains presently studied cannot be easily revealed or predicted without use of other more detailed features of distinction e.g. cell anatomy, physiology and molecular structures, if we are to gain better understanding of the differential response of those different rhizobial genera/strains (i.e. fast – vs. – slow – growers) to toxicity of copper or other antimicrobial substances.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Summary and Conclusions

The present studies showed that elevated levels of copper impacted negatively on the proliferation and survival of rhizobial strains PV_1, PV_2, CP_1 and GM_8 (objective 1). The step – wise increase in the concentration of copper did also affect nodulation and N_2 fixation of their respective symbioses both *in vitro* and *in vivo*. Generally, the toxicity effects were more pronounced in the slow – growing bradyrhizobial strains of CP_1 and GM_8 than on the fast – growing PV_1 and PV_2 (objective 2).

Also, elevated levels of copper in soil affected survival of the four rhizobial strains *in vivo*. The slow - growing strains of CP_1 and GM_8 survived more poorly in copper treated soil than the fast - growing stains. MPN studies, however, indicated that population sizes of *Rhizobium* and *Bradyrhizobium* strains in the copper contaminated soil used in the present study were not statistically different ($p = 0.05$) from those in the uncontaminated (control) soil.

The soil was thought to have a sparing effect on the toxicity of copper to rhizobia such that at concentrations of 80 ppm Cu and above, copper had more deleterious effects on rhizobia *in vitro* (proliferation in liquid media) than *in vivo* (survival in soil). Copper is known to be held on colloidal surfaces of the soil (especially on organic matter) tenaciously, thereby leading to a reduction in the intensity of copper toxicity to rhizobia.

On the basis of the present findings, it can be concluded that slow-growing rhizobia (i.e. *Bradyrhizobium*) are more susceptible to Cu toxicity both *in vitro* and *in vivo* than the fast-growing rhizobia (i.e. *Rhizobium*). It was noted, further, that there existed differences in

response to copper toxicity stress not only between the fast - and slow- growers but also between strains of the same group (fast or slow growers).

There were relatively lower rhizobial counts in the contaminated soils as compared to the control (uncontaminated) soils, although these differences were not statistically ($p = 0.05$) significant (objective 3).

5.2 Recommendations

Based on the conclusions of this study as stated above, the following are recommended:

1. Similar studies in the future should include more strains of the two rhizobial groups tested, both *in vitro* and *in vivo*.

2. A wider range of (naturally) copper-contaminated soils should be included in such future studies.

3. Physiological and/or genetic – based studies should eventually be undertaken to unequivocally explain the basis of the observed responses.

Only then, can more reliable conclusions be made regarding the differential responses to copper toxicity between and within the slow – and fast – growing rhizobia.

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APPENDICES

APPENDIX 1: Assay layout for experiment 1(a) - Effects of copper on proliferation of rhizobia/bradyrhizobia

Concentration of Cu in assay medium (µg/ml)	Volume of YEMB * (ml)	Volume of stock solution** (ml)	Volume of inoculant (ml)	Final assay volume (ml)
00	49.0	0.0	1.0	50
20	48.0	1.0	1.0	50
40	47.0	2.0	1.0	50
60	46.0	3.0	1.0	50
80	45.0	4.0	1.0	50
100	44.0	5.0	1.0	50

* YEMB= Yeast Extract Mannitol Broth

** Stock solution = 1000µg/ml$CuSO_4$

APPENDIX 2: Assay layout for experiment 1(b): Effects of copper on survival of rhizobia/bradyrhizobia

Amount of soil used (g)	CU level in assay medium (µg/g.soil)	Volume of inoculant (ml)	Plain water added (ml)	Volume of stock solution* (ml)	Final assay volume (ml)
10	00	1	2.00	0.00	3.0
10	40	1	1.96	0.04	3.0
10	80	1	1.92	0.08	3.0
10	100	1	1.90	0.10	3.0

* Stock solution = 1000µg/ml of $CuSO_4$

APPENDIX 3: Effects of copper on rhizobia/bradyrhizobia nodulation of compatible legumes *in vitro*-Assay layout for experiment 2(a)

Final copper concentration (µg/ml)	Volume of N-free nutrient solution (ml)	Volume of stock* solution added (ml)	Final assay volume (ml)
00	400	00	400
20	392	08	400
40	384	16	400
60	376	24	400
80	368	36	400
100	360	40	400

* Stock solution = 1000µg/ml$CuSO_4$

APPENDIX 4: Effects of copper on rhizobia/bradyrhizobia nodulation of compatible legumes in vivo -Assay layout for Experiment 2(b)

Final Cu concentration (µg Cu/g.soil)	Amount of soil used (g)	Amount of $CuSO_4.H_2O$ added (g)
00	3500	0.000
20	3500	0.275
40	3500	0.550
60	3500	0.825
80	3500	1.100
100	3500	1.375

APPENDIX 5: Print-out of the analysis of Variance (ANOVA) and subsequent comparison wise error rate for Population sizes *in vitro*

In this and subsequent appendices, the ranking of means (overleaf) referred to as "t –grouping" is actually the Duncan's New Multiple Range Test rankings.

Consider Two factor factorial in RCB

TINDWA Effect of soil copper accumulation on proliferation and survival of rhizobia, legume growth and process of symbio 15

Consider Two factor factorial in RCB

The GLM Procedure

Dependent Variable: pop population size (CFU/ml)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	25	882.2815178	35.2912607	1824.46	<.0001
Error	46	0.8897966	0.0193434		
Corrected Total	71	883.1713144			

R-Square	Coeff Var	Root MSE	pop Mean
0.998992	1.806658	0.139081	7.698222

Source	DF	Type I SS	Mean Square	F Value	Pr > F
block	2	0.0724667	0.0362333	1.87	0.1652
strain	3	196.8781988	65.6260663	3392.68	<.0001
CuLevel	5	420.8961819	84.1792364	4351.83	<.0001
strain*CuLevel	15	264.4346704	17.6289780	911.37	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
block	2	0.0724667	0.0362333	1.87	0.1652
strain	3	196.8781988	65.6260663	3392.68	<.0001
CuLevel	5	420.8961819	84.1792364	4351.83	<.0001
strain*CuLevel	15	264.4346704	17.6289780	911.37	<.0001

Consider Two factor factorial in RCB

The GLM Procedure

t Tests (LSD) for pop

NOTE: This test controls the Type I comparison-wise error rate, not the experiment-wise error rate.

Alpha	0.05
Error Degrees of Freedom	46
Error Mean Square	0.019343
Critical Value of t	2.01290
Least Significant Difference	0.0933

Means with the same letter are not significantly different.

t Grouping	Mean	N	strain

A	9.46133	18	PV2
B	9.23839	18	PV1
C	6.07089	18	GM8
C	6.02228	18	CP1

Test: Duncan's (Cu level x strain)
Significance Level: 0.05
Variance: 0.0193434
Degrees of Freedom: 46
n Means = 24
LSD 0.05 = 0.22858199937

Rank	Mean Name	Mean	n	Non-significant ranges
1	20	10.167	3	a
2	220	10.115	3	ab
3	10	10.01	3	abc
4	120	9.979	3	abc
5	30	9.893	3	bc
6	40	9.88	3	bc
7	420	9.826	3	cd
8	320	9.822	3	cd
9	260	9.587	3	d
10	240	9.301	3	e
11	160	9.204	3	ef
12	140	9.053	3	f
13	280	9.038	3	f
14	180	8.667	3	g
15	2100	8.56	3	g
16	440	8.519	3	g
17	1100	8.519	3	g
18	340	8.418	3	gh
19	460	8.201	3	hi
20	360	8	3	i
21	3100	0	3	j
22	380	0	3	j
23	480	0	3	j
24	4100	0	3	j

Appendix 6: Print-out of ANOVA and comparison-wise error rate for survival of rhizobia *in vivo*

Consider Repeated-Two factor factorial in RCB

The GLM Procedure

Dependent Variable: cfu colon forming unit(cells/g)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	167	6.15641667	0.03686477	12.70	<.0001
Error	192	0.55733333	0.00290278		
Corrected Total	359	6.71375000			

R-Square	Coeff Var	Root MSE	cfu Mean
0.916986	1.209596	0.053877	4.454167

Source	DF	Type I SS	Mean Square	F Value	Pr > F
strain	3	3.42230556	1.14076852	392.99	<.0001
rep	2	0.00800000	0.00400000	1.38	0.2546
CuLevel	5	0.71091667	0.14218333	48.98	<.0001
strain*CuLevel	15	0.20319444	0.01354630	4.67	<.0001
rep*strain*CuLevel	46	0.48133333	0.01046377	3.60	<.0001
time	4	0.31138889	0.07784722	26.82	<.0001
strain*time	12	0.34838889	0.02903241	10.00	<.0001
CuLevel*time	20	0.25561111	0.01278056	4.40	<.0001
strain*CuLevel*time	60	0.41527778	0.00692130	2.38	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
strain	3	3.42230556	1.14076852	392.99	<.0001
rep	2	0.00800000	0.00400000	1.38	0.2546
CuLevel	5	0.71091667	0.14218333	48.98	<.0001
strain*CuLevel	15	0.20319444	0.01354630	4.67	<.0001
rep*strain*CuLevel	46	0.48133333	0.01046377	3.60	<.0001
time	4	0.31138889	0.07784722	26.82	<.0001
strain*time	12	0.34838889	0.02903241	10.00	<.0001
CuLevel*time	20	0.25561111	0.01278056	4.40	<.0001
strain*CuLevel*time	60	0.41527778	0.00692130	2.38	<.0001

Consider Repeated-Two factor factorial in RCB

The GLM Procedure

t Tests (LSD) for cfu

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	46
Error Mean Square	0.010464
Critical Value of t	2.01290
Least Significant Difference	0.0307

Means with the same letter are not significantly different.

t Grouping	Mean	N	strain
A	4.61667	90	PV2
B	4.43444	90	GM8

	B		
B	4.40556	90	PV1
C	4.36000	90	CP1

Consider Repeated-Two factor factorial in RCB

The GLM Procedure

t Tests (LSD) for cfu
NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	192
Error Mean Square	0.002903
Critical Value of t	1.97240
Least Significant Difference	0.0177

Means with the same letter are not significantly different.

t Grouping	Mean	N	time
A	4.490278	72	4
A			
B A	4.476389	72	2
B			
B	4.461111	72	0
C	4.434722	72	6
D	4.408333	72	8

APPENDIX 7a: Print-out of ANOVA and comparison- wise error rate for effect of copper on nodule volume *in vitro*

Consider Two factor factorial in RCB

The GLM Procedure

Dependent Variable: noduleV Nodule Volume (ml)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	25	17.57146601	0.70285864	257.46	<.0001
Error	46	0.12557731	0.00272994		
Corrected Total	71	17.69704332			

R-Square	Coeff Var	Root MSE	noduleV Mean
0.992904	5.314417	0.052249	0.983153

Source	DF	Type I SS	Mean Square	F Value	Pr > F
rep	2	0.02522936	0.01261468	4.62	0.0148
strain	3	1.57820171	0.52606724	192.70	<.0001
CuLevel	5	7.63401190	1.52680238	559.28	<.0001
strain*CuLevel	15	8.33402304	0.55560154	203.52	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
rep	2	0.02522936	0.01261468	4.62	0.0148
strain	3	1.57820171	0.52606724	192.70	<.0001
CuLevel	5	7.63401190	1.52680238	559.28	<.0001
strain*CuLevel	15	8.33402304	0.55560154	203.52	<.0001

Consider Two factor factorial in RCB

The GLM Procedure

t Tests (LSD) for noduleV

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	46
Error Mean Square	0.00273
Critical Value of t	2.01290
Least Significant Difference	0.0351

Means with the same letter are not significantly different.

t Grouping	Mean	N	strain
A	1.13883	18	PV2
B	1.08933	18	PV1
C	0.94678	18	CP1
D	0.75767	18	GM8

Test: Duncan's (Cu level x strain)
Significance Level: 0.05
Variance: 0.00272994
Degrees of Freedom: 46
Keep If:

n Means = 24
LSD 0.05 = 0.08587211742

	Rank	Mean	Name	Mean	n	Non-significant ranges
1	30	2.633			3	a
2	320	1.367			3	b
3	40	1.367			3	b
4	260	1.233			3	c
5	10	1.2			3	cd
6	20	1.2			3	cd
7	220	1.2			3	cd
8	120	1.167			3	cde
9	2100	1.133			3	def
10	140	1.133			3	def
11	240	1.1			3	efg
12	440	1.067			3	fgh
13	180	1.033			3	ghi
14	160	1.02			3	ghi
15	1100	0.983			3	hi
16	280	0.967			3	i
17	460	0.833			3	j
18	420	0.813			3	j
19	340	0.6			3	k
20	360	0.467			3	l
21	480	0.333			3	m
22	380	0.3137			3	m
23	3100	0.3			3	m
24	4100	0.133			3	n

APPENDIX 7b: Print-out of ANOVA and comparison – wise error rate for effect of copper on nodule volume *in vivo*

Consider Two factor factorial in RCB

The GLM Procedure

Dependent Variable: noduleV Nodule Volume (ml)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	25	24.36051806	0.97442072	29.71	<.0001
Error	46	1.50888056	0.03280175		
Corrected Total	71	25.86939861			

R-Square	Coeff Var	Root MSE	noduleV Mean
0.941673	4.254103	0.181113	4.257361

Source	DF	Type I SS	Mean Square	F Value	Pr > F
block	2	0.02978611	0.01489306	0.45	0.6379
strain	3	2.17484861	0.72494954	22.10	<.0001
CuLevel	5	17.45349028	3.49069806	106.42	<.0001
strain*CuLevel	15	4.70239306	0.31349287	9.56	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
block	2	0.02978611	0.01489306	0.45	0.6379
strain	3	2.17484861	0.72494954	22.10	<.0001
CuLevel	5	17.45349028	3.49069806	106.42	<.0001
strain*CuLevel	15	4.70239306	0.31349287	9.56	<.0001

Consider Two factor factorial in RCB

The GLM Procedure

t Tests (LSD) for noduleV

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	46
Error Mean Square	0.032802
Critical Value of t	2.01290
Least Significant Difference	0.1215

Means with the same letter are not significantly different.

t Grouping	Mean	N	strain
A	4.52278	18	PV1
B	4.30278	18	PV2
C	4.10389	18	GM8
C	4.10000	18	CP1

Test: Duncan's (Cu level x strain)
Significance Level: 0.05
Variance: 0.03280175
Degrees of Freedom: 46
Keep If:

n Means = 24
LSD 0.05 = 0.29766250202

Rank	Mean Name	Mean	n	Non-significant ranges
1	30	4.98	3	a
2	10	4.956	3	a
3	40	4.847	3	ab
4	340	4.787	3	abc
5	320	4.763	3	abc
6	420	4.727	3	abcd
7	440	4.724	3	abcd
8	20	4.713	3	abcd
9	140	4.613	3	bcde
10	160	4.597	3	bcde
11	120	4.587	3	bcde
12	240	4.45	3	cdef
13	220	4.413	3	defg
14	180	4.283	3	efg
15	260	4.25	3	fg
16	280	4.133	3	fgh
17	1100	4.103	3	gh
18	2100	3.857	3	hi
19	460	3.773	3	i
20	360	3.763	3	i
21	480	3.456	3	j
22	380	3.277	3	jk
23	4100	3.039	3	k
24	3100	3.03	3	k

APPENDIX 8a: Print-out of ANOVA and comparison-wise error rate for effect of copper on fresh nodule mass *in vitro*

Consider Two factor factorial in RCB

The GLM Procedure

Dependent Variable: noduleM Nodule mass (g)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	25	6388094.625	255523.785	4866.62	<.0001
Error	46	2415.250	52.505		
Corrected Total	71	6390509.875			

R-Square	Coeff Var	Root MSE	noduleM Mean
0.999622	1.400093	7.246063	517.5417

Source	DF	Type I SS	Mean Square	F Value	Pr > F
rep	2	1186.083	593.042	11.29	0.0001
strain	3	2502150.375	834050.125	15885.0	<.0001
CuLevel	5	2990809.792	598161.958	11392.4	<.0001
strain*CuLevel	15	893948.375	59596.558	1135.06	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
rep	2	1186.083	593.042	11.29	0.0001
strain	3	2502150.375	834050.125	15885.0	<.0001
CuLevel	5	2990809.792	598161.958	11392.4	<.0001
strain*CuLevel	15	893948.375	59596.558	1135.06	<.0001

Consider Two factor factorial in RCB

The GLM Procedure

t Tests (LSD) for noduleM

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	46
Error Mean Square	52.50543
Critical Value of t	2.01290
Least Significant Difference	4.8619

Means with the same letter are not significantly different.

t Grouping	Mean	N	strain
A	703.167	18	PV1
A	701.500	18	PV2
B	367.500	18	CP1

C 298.000 18 GM8

Test: Duncan's (cu level x strain)
Significance Level: 0.05
Variance: 52.505
Degrees of Freedom: 46
Keep If:

n Means = 24
LSD 0.05 = 11.9090180138

Rank	Mean	Name	Mean	n	Non-significant ranges
1	30	1131	3	a	
2	20	953	3	b	
3	10	953	3	b	
4	120	803	3	c	
5	220	783	3	d	
6	140	750	3	e	
7	240	742	3	ef	
8	160	737	3	f	
9	260	713	3	g	
10	320	553	3	h	
11	280	547	3	h	
12	40	533	3	i	
13	180	513	3	j	
14	1100	473	3	k	
15	2100	470	3	k	
16	420	407	3	l	
17	440	343	3	m	
18	460	247	3	n	
19	340	170	3	o	
20	480	170	3	o	
21	360	158	3	o	
22	380	128	3	p	
23	4100	87	3	q	
24	3100	65	3	r	

APPENDIX 8b: Print-out of ANOVA and comparison-wise error rate for effect of copper on nodule mass *in vivo*

Consider Two factor factorial in RCB

The GLM Procedure

Dependent Variable: nodule M Nodule mass (g)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	25	10.51649251	0.42065970	294.09	<.0001
Error	46	0.06579847	0.00143040		
Corrected Total	71	10.58229099			

R-Square	Coeff Var	Root MSE	noduleM Mean
0.993782	6.041771	0.037821	0.625986

Source	DF	Type I SS	Mean Square	F Value	Pr > F
block	2	0.00324553	0.00162276	1.13	0.3304
strain	3	5.27905671	1.75968557	1230.20	<.0001
CuLevel	5	0.70090674	0.14018135	98.00	<.0001
strain*CuLevel	15	4.53328354	0.30221890	211.28	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
block	2	0.00324553	0.00162276	1.13	0.3304
strain	3	5.27905671	1.75968557	1230.20	<.0001
CuLevel	5	0.70090674	0.14018135	98.00	<.0001
strain*CuLevel	15	4.53328354	0.30221890	211.28	<.0001

Consider Two factor factorial in RCB

The GLM Procedure

t Tests (LSD) for noduleM

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	46
Error Mean Square	0.00143
Critical Value of t	2.01290
Least Significant Difference	0.0254

Means with the same letter are not significantly different.

t Grouping	Mean	N	strain
A	1.09333	18	CP1
B	0.50667	18	PV1
C	0.45783	18	GM8
C	0.44611	18	PV2

Test: Duncan's (Cu level x Strain)
Significance Level: 0.05
Variance: 0.0014304
Degrees of Freedom: 46
Keep If:

n Means = 24
LSD 0.05 = 0.06215905045

Rank	Mean Name	Mean	n	Non-significant ranges
1	30	1610	3	a
2	320	1607	3	b
3	40	1172	3	c
4	420	1159	3	d
5	440	1110	3	e
6	340	1073	3	f
7	460	953	3	g
8	360	920	3	h
9	480	893	3	i
10	380	890	3	j
11	10	577	3	k
12	120	547	3	l
13	160	503	3	m
14	140	490	3	n
15	1100	467	3	o
16	220	467	3	o
17	240	467	3	o
18	20	467	3	o
19	3100	460	3	p
20	4100	460	3	p
21	180	457	3	q
22	260	437	3	r
23	280	430	3	s
24	2100	410	3	t

APPENDIX 9a: Printout of ANOVA and comparison wise error rate for effect of copper on shoot total nitrogen *in vitro*

Consider Two factor factorial in RCB

The GLM Procedure

Dependent Variable: TN Total nitrogen (%)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	25	14.22900833	0.56916033	144.86	<.0001
Error	46	0.18074167	0.00392917		
Corrected Total	71	14.40975000			

R-Square	Coeff Var	Root MSE	TN Mean
0.987457	3.455199	0.062683	1.814167

Source	DF	Type I SS	Mean Square	F Value	Pr > F
rep	2	0.13505833	0.06752917	17.19	<.0001
strain	3	4.99815000	1.66605000	424.02	<.0001
CuLevel	5	4.59280000	0.91856000	233.78	<.0001
strain*CuLevel	15	4.50300000	0.30020000	76.40	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
rep	2	0.13505833	0.06752917	17.19	<.0001
strain	3	4.99815000	1.66605000	424.02	<.0001
CuLevel	5	4.59280000	0.91856000	233.78	<.0001
strain*CuLevel	15	4.50300000	0.30020000	76.40	<.0001

Consider Two factor factorial in RCB

The GLM Procedure

t Tests (LSD) for TN

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	46
Error Mean Square	0.003929
Critical Value of t	2.01290
Least Significant Difference	0.0421

Means with the same letter are not significantly different.

t Grouping	Mean	N	strain
A	2.12667	18	GM8
B	2.02333	18	CP1
C	1.56333	18	PV1
C	1.54333	18	PV2

Test: Duncan's (cu level x strain)
Significance Level: 0.05
Variance: 0.00392917
Degrees of Freedom: 46
Keep If:

n Means = 24
LSD 0.05 = 0.10302106389

Rank	Mean Name	Mean	n	Non-significant ranges
1	30	3.23	3	a
2	320	2.59	3	b
3	40	2.57	3	b
4	420	2.28	3	c
5	440	2.14	3	d
6	460	2.03	3	e
7	480	1.91	3	f
8	4100	1.83	3	fg
9	340	1.74	3	gh
10	10	1.64	3	hi
11	120	1.63	3	i
12	20	1.62	3	ij
13	360	1.61	3	ij
14	220	1.61	3	ij
15	380	1.58	3	ijk
16	160	1.56	3	ijkl
17	240	1.55	3	ijkl
18	140	1.55	3	ijkl
19	260	1.53	3	ijkl
20	180	1.53	3	ijkl
21	280	1.5	3	jklm
22	1100	1.47	3	klm
23	2100	1.45	3	lm
24	3100	1.39	3	m

APPENDIX 9b: Print-out of ANOVA and comparison-wise error rate for effect of copper on nodule shoot total nitrogen *in vivo*

Consider Two factor factorial in RCB

The GLM Procedure

Dependent Variable: TN Total nitrogen (%)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	25	24.36051806	0.97442072	29.71	<.0001
Error	46	1.50888056	0.03280175		
Corrected Total	71	25.86939861			

R-Square	Coeff Var	Root MSE	TN Mean
0.941673	4.254103	0.181113	4.257361

Source	DF	Type I SS	Mean Square	F Value	Pr > F
block	2	0.02978611	0.01489306	0.45	0.6379
strain	3	2.17484861	0.72494954	22.10	<.0001
CuLevel	5	17.45349028	3.49069806	106.42	<.0001
strain*CuLevel	15	4.70239306	0.31349287	9.56	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
block	2	0.02978611	0.01489306	0.45	0.6379
strain	3	2.17484861	0.72494954	22.10	<.0001
CuLevel	5	17.45349028	3.49069806	106.42	<.0001
strain*CuLevel	15	4.70239306	0.31349287	9.56	<.0001

Consider Two factor factorial in RCB

The GLM Procedure

t Tests (LSD) for TN

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	46
Error Mean Square	0.032802
Critical Value of t	2.01290
Least Significant Difference	0.1215

Means with the same letter are not significantly different.

t Grouping	Mean	N	strain
A	4.52278	18	PV1
B	4.30278	18	PV2
C	4.10389	18	CP1
C	4.10000	18	GM8

Test: Duncan's
Significance Level: 0.05
Variance: 0.03280175
Degrees of Freedom: 46
Keep If:

n Means = 24
LSD 0.05 = 0.29766250202

Rank	Mean	Name	Mean	n	Non-significant ranges
1	40	4.98	3	a	
2	10	4.95	3	a	
3	30	4.85	3	ab	
4	440	4.79	3	ab	
5	420	4.76	3	abc	
6	320	4.74	3	abcd	
7	340	4.73	3	abcd	
8	20	4.71	3	abcd	
9	140	4.6	3	bcde	
10	160	4.59	3	bcde	
11	120	4.59	3	bcde	
12	240	4.44	3	cdef	
13	220	4.41	3	defg	
14	180	4.28	3	efg	
15	260	4.25	3	fg	
16	280	4.13	3	fgh	
17	1100	4.1	3	gh	
18	2100	3.87	3	hi	
19	360	3.77	3	i	
20	460	3.76	3	i	
21	380	3.45	3	j	
22	480	3.28	3	jk	
23	3100	3.09	3	k	
24	4100	3.03	3	k	

Appendix 10: A print-out of ANOVA and comparison–wise error rate for MPN counts in contaminated and uncontaminated soils

Analysis of variance

Variate: MPN

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Strain_Type	3	26484617.	8828206.	16.08	<.001
Cu_level	1	1111982.	1111982.	2.03	0.174
Strain_Type.Cu_level	3	306570.	102190.	0.19	0.904
Residual	16	8784803.	549050.		
Total	23	36687972.			

***** Tables of means *****

Variate: MPN

Grand mean 2545.

Strain_Type	Cowp	Soybean	Bean	Bean
	3867.	3281.	1457.	1575.

Cu_level	Contam	Uncon.
	2760.	2330.

Strain_Type	Cu_level	Conta.	Uncon.
Cowp		3932	3802
Soyb		3437	3125
Bean		1816	1097
Bean		1855	1295

*** Standard errors of means ***

Table	Strain_Type	Cu_level	Strain_Type Cu_level
rep.	6	12	3
d.f.	16	16	16
e.s.e.	302.5	213.9	427.8

*** Standard errors of differences of means ***

Table	Strain_Type	Cu_level	Strain_Type Cu_level
rep.	6	12	3
d.f.	16	16	16
s.e.d.	427.8	302.5	605.0

c

*** Least significant differences of means (5% level) ***

Table	Strain_Type	Cu_level	Strain_Type Cu_level
rep.	6	12	3
d.f.	16	16	16
l.s.d.	906.9	641.3	1282.6

All pairwise comparisons are tested.

Variance = 549050.2083 with 16 degrees of freedom

Duncan's multiple range test

Experimentwise error rate = 0.0500
Comparisonwise error rates

Mean	vs	Mean	t	significant
3		4	-0.277	No
3		2	-4.264	Yes
3		1	-5.634	Yes
4		2	-3.987	Yes
4		1	-5.357	Yes
2		1	-1.370	No

Identifier	Mean
Cowpea rhizobia	1457a
Soybean rhizobia	1575a
Bean rhizobia	3281b
Bean rhizobia	3867b

Appendix 11: A printout of the means for the interaction (Strain X Cu level) effects for the experiments corresponding to Tables 2, 4, 6, and 8 in the text

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Consider Two factor factorial in RCB

The GLM Procedure

Level of strain	Level of CuLevel	N	pop Mean	pop Std Dev	noduleV Mean	noduleV Std Dev	noduleM Mean	noduleM Std Dev	TN Mean	TN Std Dev
CP1	0	3	9.8933333	0.09504385	4.98000000	0.02645751	1.61000000	0.08185353	4.84666667	0.10115994
CP1	20	3	9.8220000	0.04708503	4.76333333	0.18502252	1.60666667	0.08962886	4.73666667	0.08621678
CP1	40	3	8.4183333	0.10161365	4.78666667	0.21571586	1.07333333	0.06429101	4.72333333	0.13868429
CP1	60	3	8.0000000	0.00000000	3.76333333	0.19399313	0.92000000	0.02645751	3.77333333	0.15947832
CP1	80	3	0.0000000	0.00000000	3.27666667	0.05033223	0.89000000	0.01732051	3.45000000	0.05567764
CP1	100	3	0.0000000	0.00000000	3.03000000	0.07810250	0.46000000	0.02645751	3.09333333	0.09609024
GM8	0	3	9.8800000	0.01732051	4.84666667	0.10115994	0.17166667	0.01357694	4.98000000	0.02645751
GM8	20	3	9.8260000	0.01682260	4.73666667	0.08621678	0.15866667	0.00208167	4.76333333	0.18502252
GM8	40	3	8.5186667	0.07216878	4.72333333	0.13868429	0.11000000	0.01732051	4.78666667	0.21571586
GM8	60	3	8.2006667	0.17378243	3.77333333	0.15947832	0.95333333	0.04041452	3.76333333	0.19399313
GM8	80	3	0.0000000	0.00000000	3.45000000	0.05567764	0.89333333	0.03214550	3.27666667	0.05033223
GM8	100	3	0.0000000	0.00000000	3.09333333	0.09609024	0.46000000	0.02000000	3.03000000	0.07810250
PV1	0	3	10.0100000	0.02000000	4.95333333	0.14364308	0.57666667	0.01154701	4.95333333	0.14364308
PV1	20	3	9.9790000	0.00519615	4.58666667	0.25146239	0.54666667	0.02081666	4.58666667	0.25146239
PV1	40	3	9.0526667	0.04561067	4.61333333	0.26083200	0.49000000	0.01732051	4.61333333	0.26083200
PV1	60	3	9.2033333	0.63002407	4.59666667	0.28676355	0.50333333	0.06027714	4.59666667	0.28676355
PV1	80	3	8.6666667	0.05600298	4.28333333	0.27682726	0.45666667	0.02516611	4.28333333	0.27682726
PV1	100	3	8.5186667	0.07216878	4.10333333	0.30105371	0.46666667	0.00577350	4.10333333	0.30105371
PV2	0	3	10.1666667	0.06110101	4.71333333	0.14977761	0.46666667	0.00577350	4.71333333	0.14977761
PV2	20	3	10.1156667	0.03175426	4.41333333	0.19139836	0.46666667	0.01527525	4.41333333	0.19139836
PV2	40	3	9.3006667	0.02150194	4.45000000	0.02645751	0.46666667	0.00577350	4.45000000	0.02645751
PV2	60	3	9.5873333	0.01270171	4.25000000	0.23895606	0.43666667	0.04163332	4.25000000	0.23895606
PV2	80	3	9.0373333	0.07216878	4.13333333	0.16921387	0.43000000	0.03605551	4.13333333	0.16921387
PV2	100	3	8.5603333	0.07216878	3.85666667	0.14153916	0.41000000	0.04358899	3.85666667	0.1415391

Appendix 12: A printout of the means for the interaction (Strain X Cu level) effects for the experiments corresponding to Tables 3, 5, and 7 in the text

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Consider Two factor factorial in RCB

The GLM Procedure

Level of strain	Level of CuLevel	N	noduleV Mean	noduleV Std Dev	noduleM Mean	noduleM Std Dev	TN Mean	TN Std Dev
CP1	0	3	2.63300000	0.01700000	1131.00000	8.5440037	3.23000000	0.07000000
CP1	20	3	1.36700000	0.17305202	553.00000	25.2388589	2.59000000	0.06557439
CP1	40	3	0.60000000	0.10000000	170.00000	4.0000000	1.74000000	0.05196152
CP1	60	3	0.46700000	0.00100000	158.00000	1.0000000	1.61000000	0.10535654
CP1	80	3	0.31366667	0.03784618	128.00000	6.2449980	1.58000000	0.00000000
CP1	100	3	0.30000000	0.05000000	65.00000	3.0000000	1.39000000	0.06082763
GM8	0	3	1.36700000	0.02700000	533.00000	1.0000000	2.57000000	0.00000000
GM8	20	3	0.81300000	0.00264575	407.00000	3.0000000	2.28000000	0.01732051
GM8	40	3	1.06700000	0.00173205	343.00000	3.4641016	2.14000000	0.23579652
GM8	60	3	0.83300000	0.01300000	247.00000	2.0000000	2.03000000	0.04358899
GM8	80	3	0.33300000	0.00300000	170.00000	10.4403065	1.91000000	0.01000000
GM8	100	3	0.13300000	0.00100000	88.00000	6.2449980	1.83000000	0.07000000
PV1	0	3	1.20000000	0.00300000	953.00000	3.0000000	1.64000000	0.02000000
PV1	20	3	1.16700000	0.00984886	803.00000	11.2694277	1.63000000	0.18520259
PV1	40	3	1.13300000	0.00818535	750.00000	2.0000000	1.55000000	0.05000000
PV1	60	3	1.02000000	0.00916515	727.00000	8.1853528	1.56000000	0.06928203
PV1	80	3	1.03300000	0.02586503	513.00000	14.7986486	1.53000000	0.10440307
PV1	100	3	0.98300000	0.00300000	473.00000	7.8102497	1.47000000	0.01000000
PV2	0	3	1.20000000	0.00556776	953.33333	2.0816660	1.62000000	0.01000000
PV2	20	3	1.20000000	0.13843410	783.00000	12.1655251	1.61000000	0.09539392
PV2	40	3	1.10000000	0.10000000	742.00000	1.0000000	1.55000000	0.05000000
PV2	60	3	1.23300000	0.00100000	713.66667	8.0208063	1.53000000	0.00000000
PV2	80	3	0.96700000	0.01300000	547.00000	10.5356538	1.50000000	0.01000000
PV2	100	3	1.13300000	0.00400000	470.00000	6.0000000	1.45000000	0.06000000