THE IMPACT OF COPPER ON FILAMENTOUS FUNGI AND YEASTS PRESENT IN SOIL

By

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Thesis presented in partial fulfillment of the requirements for the degree of Master of Science at the University of Stellenbosch.

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April 2004

Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for degree.

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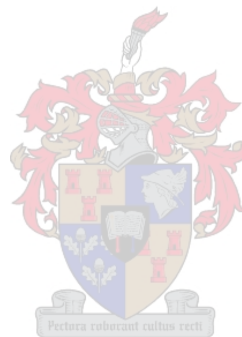

SUMMARY

Numerous workers studied the impact of pollutants and agricultural chemicals, containing heavy metals such as copper (Cu), on soil microbes. It was found that elevated soil Cu levels do have a detrimental effect on soil bacterial populations however the filamentous fungi seemed to be less affected. Most of these studies were conducted in soils containing already relatively high Cu levels and the effect of this heavy metal on the non-filamentous fungi (i.e. yeasts) was never investigated. The aim of this study was therefore to determine the impact of elevated Cu levels on filamentous fungi and yeasts occurring in soils containing relatively low natural Cu levels. A synthetic selective medium containing glucose as carbon source, thymine as nitrogen source, vitamins, minerals and chloramphenicol as anti-bacterial agent (TMV-agar), was used to enumerate ascomycetous and basidiomycetous Cu resistant yeasts in a sample of virgin soil containing ~ 2ppm Cu. Media that were used to enumerate Cu resistant filamentous fungi were malt extract agar, malt extract agar with streptomycin sulfate, malt-yeast-extract-peptone agar with chloramphenicol and streptomycin sulfate, benomyl–dichloran-streptomycin medium for the enumeration of hymenomycetous fungi and two selective media for the isolation of mucoralean fungi. Cu resistant fungi able to grow on all of the above mentioned solid media supplemented with 32 ppm Cu occurred in the soil sample. To obtain an indication of the level of Cu tolerance of fungi present in this soil sample, a number of fungal isolates were screened for the ability to grow on a series of agar plates, prepared from glucose-glutamate-yeast extract agar, containing increasing concentrations of Cu. It was found that filamentous fungi and yeasts that were able to grow on this agar medium containing up to 100 ppm Cu were present in the soil. A series of soil microcosms was subsequently prepared from the soil sample by experimentally contaminating the soil with increasing amounts of copper oxychloride, were after fungal populations in the microcosms, including Cu resistant fungi, were monitored using plate counts. At the end of the incubation period, after 245 days, fungal biomass in the microcosms was compared by determining the concentrations of the fungal sterol, ergosterol, in

the soil. Generally, Cu had little impact on the numbers of filamentous fungal colony forming units on the plates, as well as on the ergosterol content of the soil. The numbers of filamentous fungi in the soil, including the Mucorales and hymenomycetes, seemed to be less affected by the addition of copper oxychloride than the numbers of soil yeasts able to grow on TMV-agar. The focus of the next chapter was on the response of yeasts in different soils to elevated levels of Cu in the soil. TMV-agar was used to enumerate yeasts in soil microcosms prepared from four different soil samples, which were experimentally treated with copper oxychloride resulting in Cu concentrations of up to 1000 ppm. The selective medium supplemented with 32 ppm Cu was used to enumerate Cu resistant yeasts in the microcosms. The results showed that the addition of Cu at concentrations ≥ ~1000 ppm did not have a significant effect on total yeast numbers in the soil. Furthermore, it was found that Cu resistant yeasts were present in all the soil samples regardless of the amount of Cu that the soil was challenged with. At the end of the incubation period, yeasts in the microcosms with zero and ~1000 ppm additional Cu were enumerated, isolated and identified using sequence analyses of the D1/D2 600-650bp region of the large subunit of ribosomal DNA. Hymenomycetous species dominated in the control soil, while higher numbers of the urediniomycetous species were found in the soil that received Cu. These observations suggest that urediniomycetous yeasts may play an important role in re-establishing overall microbial activity in soils following perturbations such as the addition of Cu-based fungicides.

Key words: Copper, fungi, heavy metals, soil, yeasts.

OPSOMMING

Vele navorsers het al die impak van besoedelingstowwe en landbou-chemikalieë wat swaarmetale soos koper (Cu) bevat, op grond-mikrobes bestudeer. Dit is gevind dat verhoogde Cu vlakke ‘n nadelige effek het op grond-bakteriese populasies, maar dat die filamentagtige fungi geneig is om minder geaffekteer te word. Meeste van hierdie studies is gedoen met gronde wat alreeds relatief hoë Cu vlakke bevat het en die effek van hierdie swaarmetaal op die nie-filamentagtige fungi (d.i. giste) is nooit ondersoek nie. Die doel van hierdie studie was dus om die impak van verhoogde Cu vlakke op filamentagtige fungi en giste in gronde, wat natuurlike lae vlakke van Cu bevat, te bepaal. ‘n Sintetiese selektiewe medium wat glukose as koolstofbron, timien as stikstofbron, vitamiene, minerale asook chloramfenikol as anti-bakteriese agent bevat (TMV-agar), is gebruik om askomisete en basidiomisete Cu weerstandbiedende giste in ‘n monster ongeskonde grond, bevattende ~ 2dpm Cu, te tel. Media wat gebruik is om Cu weerstandbiedende filamentagtige fungi te tel, was mout-ekstrak agar, mout-ekstrak agar met streptomisiensulfaat, benomiel-dichloran-streptomisien medium vir die tel van hiemenomiseetagtige fungi en twee media vir die isolasie van mukoraliese fungi. Cu-weerstandbiedende fungi wat op al die bogenoemde media, aangevul met 32 dpm Cu, kon groei, het in die grondmonster voorgekom. Om die mate van Cu-weerstandbiedendheid van fungi wat in die grondmonster voorkom, te bepaal, is ‘n getal fungus-isolate op agarplate, voorberei met glukose-glutamaat-gis ekstrak agar, bevattende verhoogde konsentrasies Cu, nagegaan. Daar is gevind dat daar filamentagtige fungi en giste in die grond voorkom wat die vermoë het om op media bevattende 100 dpm Cu te groei. ‘n Reeks grond mikrokosmosse is dus voorberei vanaf die grondmonster deur om dit eksperimenteel te kontamineer met verhoogde hoeveelhede koper oksichloried, waarna die fungus-populasies asook die Cu-weerstandbiedende fungi in die mikrokosmos gemoniteer is deur middel van plaattellings. Aan die einde van die inkubasie periode, 245 dae, is die fungus biomassa in al die mikrokosmosse bereken deur die konsentrasie van die fungus sterool ergosterool te bepaal en dit met mekaar te vergelyk. Oor die algemeen het Cu min impak ten opsigte van die

getal filamentagtige fungi kolonie vormende eenhede die plate, asook op die ergosterool inhoud van die grond gehad. Dit wil voorkom of die getal filamentagtige fungi in die grond, insluitende die Mucorales en die hymenomisete, minder geaffekteer is deur die toediening van koperoksichloried as die aantal grondgiste wat op die TMV-agar kan groei. Die fokus van die volgende hoofstuk was dus op die reaksie wat giste in verskillende grondtipes gehad het op verhoogde Cu in die grond. TMV-agar is gebruik om die getal giste te bepaal in die grond mikrokosmosse van die vier verskillende grondmonsters, wat voorberei is deur om dit eksperimenteel met koper oksikloried te kontamineer tot en met Cu konsentrasies van 1000 dpm. Die selektiewe medium wat gesupplementeer is met 32 dpm Cu, is gebruik om Cu weerstandbiedende giste in die mikrokosmosse te bepaal. Die resultate toon dat die toevoeging van Cu by konsentrasies $\geq$ ~1000 dpm nie enige beduidende effek op die totale gis getalle gehad het nie. Daar is ook gevind dat daar Cu weerstandbiedende giste in die grond monsters voorkom gekom het ten spyte van die hoeveelheid Cu wat tot die grond toegevoeg is. Aan die einde van die inkubasie periode is die giste wat die die mikrokosmosse bevattende nul en ~1000 dpm Cu getel, geïsoleer en geïdentifiseer deur gebruik te maak van DNA volgorde bepaling van die D1/D2 600-650 bp areas geleë in die groter subeenheid van die ribosonale DNA. Hymenomisete spesies het in die grond kontrole gedomineer, terwyl hoër getalle uredinomisete spesies in die grond met addisionele Cu gevind is. Die resultate dui daarop dat uredinomisete giste dalk ‘n belangrike rol kan speel in die hervestiging van die oorwegende mikrobiese aktiwiteit in grond na skoktoestande soos die aanwending van Cu-gebaseerde fungisiede.

Sleutelwoorde: Koper, fungi, swaarmetale, grond en giste.

ACKNOWLEDGEMENTS

I would like to express my appreciation to the following:

My supervisors, Proff A. Botha and G.M. Wolfaardt, for their guidance, advice and valuable contribution they made towards this study, especially Prof Botha, for the many hours he spent helping and supporting me.

Dr. WJ. Conradie and Mr. P. Olivier of ARC-Nietvoorbij for the technical support.

Dr. CH. Pohl of the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, who conducted the soil sterol analysis.

Winetech and the National Research Foundation for funding.

The staff and students of the Department of Microbiology at the University of Stellenbosch for their support and advice, especially the students of the Botha-lab.

And last for my mom, family, friends and flatmates, for the constant love, support and encouragement throughout the study.

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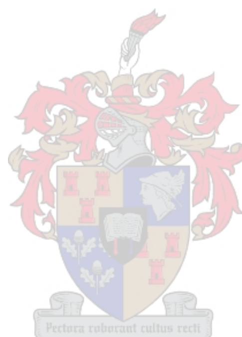

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Chapter 1

Literature Review

1.1. BACKGROUND

In the Western Cape, South Africa, the level of copper (Cu) in some soils may be as low as 0.1 ppm (Conradie, 1999). Although some of this Cu may be of natural origin, it was found that soil Cu concentrations might also be affected by agricultural practices (Loneragan *et al*., 1981). In these cases, Cu reaches the soil as a component of fertilizers and/or fungicides.

Even though Cu is an important micronutrient of most microbes, it could act as an inhibitor of microbial growth at high concentrations (Gadd, 1993), and may even change the metabolic profile of soil (Duxbury, 1985). For instance, bacterial numbers in soil were found to decrease after Cu application (Bååth *et al*., 1998). In contrast, filamentous fungi were found to be less susceptible to elevated soil Cu levels than prokaryotes (Arnebrant *et al*., 1987; Doelman, 1985; Hiroki, 1992). However, relatively little is known about the impact of Cu on the yeast populations in soil.

With the above as background, the aim of this study was to determine whether indigenous fungi of the Western Cape, including yeasts occurring in soil containing relatively low Cu concentrations, also have the ability to survive and grow in the presence of high Cu concentrations. In this study the focus was mostly on the response of yeasts in different soils to elevated levels of Cu in the soil. Such information is relevant especially in wine-producing regions where Cu-based fungicides are widely used.

To achieve these goals the following experiments were conducted as described in Chapter 2: Firstly, soil dilution plates were prepared from different soil samples to determine the proportion of ascomycetous and basidiomycetous yeasts that were able to grow on a selective medium described by Mothibeli (1996). The medium was then used to enumerate a diverse group of unrelated soil yeasts. A sample from virgin soil, containing a low natural Cu concentration, was subsequently investigated for the presence of filamentous fungi and yeasts able to grow on a series of different solid media containing 32 ppm Cu. To obtain an indication of the level of Cu tolerance of fungi present in this soil sample, fungal

isolates were screened for the ability to grow on a series of agar plates containing increasing concentrations of Cu. In addition, fungi in a series of soil microcosms prepared by experimentally contaminating the virgin soil mentioned above with increasing amounts of Cu were compared on the basis of plate counts and analysis of the fungal sterol, ergosterol, in the soil.

Subsequently, the medium described by Mothibeli (1996) was used to monitor a diverse group of unrelated yeasts in soil microcosms prepared from four different soil samples, which were experimentally treated with the fungicide, copper oxychloride (Chapter 3).

1.2. THE CHEMISTRY OF COPPER

1.2.1. The physical and chemical characteristics of copper

Copper (Cu) is a native element that occurs naturally in rocks and soils (Baker and Senft, 1995). It is also known as a noble element, since it is classified in sub-group 1B, the gold group on the Periodic Table, which comprises, gold (Au), silver (Ag) and Cu (West, 1982). Cu, in its metal state, has a distinctive salmon red colour, making it one of the few metals that are coloured. Its alloys can vary in colour from reddish yellow to even purple. A green colour is transmitted when pure white light is projected through Cu foil less than 0.025 μm thick.

The nucleus of the Cu atom contains 29 neutrons and protons, with 29 electrons distributed as $1s^2$, $2s^2$, $2p^6$, $3s^2$, $3p^6$, $3d^{10}$, $4s^1$. It has two natural occurring isotopes, Cu^{63} (69.09 %) and Cu^{65} (30.01 %), as well as three artificial isotopes that are listed in Table 1.1 (Loneragan, 1981).

Cu has a density of 8.932 g/cm^3 at 20°C, but it can vary according to the history of the metal, especially the oxygen content (West, 1982). At melting point the density can drop to 8.32 g/cm^3, with liquid Cu being at 7.99 g/dm^3. However, since this density is still greater than 6 g/cm^3 it is classified as a heavy metal (Baker and Senft, 1995).

The melting point of Cu is 1083°C and the boiling temperature is 2595°C (Wilson, 1998). Cu's thermal conductivity (400 W/mK) is higher than any other commonly available metal, but can also reduce rapidly with additions of other metals. Although the electrical conductivity rapidly rises with lowering of the temperature, thermal conductivity varies less dramatically. Above-mentioned properties along with its non-magnetic nature are the most significant properties of this heavy metal. This is the prime reason why it has been chosen as the standard of 100% conductivity. This is known as the International Annealed Copper Standard (IACS), which is equivalent to an electrical resistance of 0.017241Ω mm^2/m at 20°C. The maximum theoretical conductivity of Cu is at 103.4% or 1.667Ωm.

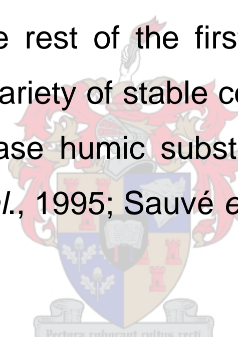
Table 1.1. Atomic and nuclear properties of Cu (West, 1982).

Property		Value
Atomic number		29
Atomic weight (mass)		63.54
Atomic radius		1.275 Å
Valency		1 and 2
Ionic radius:	Cu^{2+}	0.72 Å
	Cu^{1+}	0.96 Å
Ionisation potential:	Cu^{2+}	20.30 v
	Cu^{1+}	7.72 v
Neutron absorption cross-section at 2200 m/s		3.85± 0.12 barns/atoms
Artificial Isotopes:	Cu^{64} Half life	12.84 h
	Cu^{66} Half life	5.20 min
	Cu^{67} Half life	61.00 h

Cu's chemical characteristics make it suitable for a wide range of uses (West, 1982). It can interact with a large amount of other elements forming bronzes, brasses and minerals. It does not corrode easily, making it a rather durable metal. At room temperature, oxidation occurs very slowly, but as the temperature increases, oxidation becomes more rapidly. Cu does not form a hydride, but

under molten conditions it can dissolve hydrogen. Even though Cu does not react with diluted sulphuric and organic acids, it rapidly reacts with nitric and hydrochloric acids in the absence of oxygen or air. It also reacts less vigorously with phosphoric and hydrofluoric acids. It is not resistant to clays and loam in soil and it may corrode in defined soil.

As previously mentioned, the electronic structure of a free Cu atom is $1s^2$, $2s^2$, $2p^6$, $3s^2$, $3p^6$, $3d^{10}$, $4s^1$ (Parker, 1981). Thus the single 4s electron is outside a filled 3d shell and is rather difficult to remove from the Cu atom to form a Cu^{1+} ion. Like all other elements in the first transition series, two electrons are more easily removed to form Cu^{2+}. This ion is very stable in water. Cu's second ionisation potential is much higher than the first and thus the effect of the environment of the ion allow a stable Cu^{1+} to exist. Whether Cu exists in the Cu^{1+} or Cu^{2+} species depends on the physical environment, solvent, concentration and which ligands (bases) are present. Like the rest of the first transition series on the Periodic Table, Cu is known to form a variety of stable complexes with bases and chelating agents like EDTA or solid-phase humic substances, for instance humic acid in soils (Alloway, 1995; Hong *et al.*, 1995; Sauvé *et al.*, 1998).



1.3. SOIL

1.3.1. The physical and chemical characteristics of soil

Soil is a complicated system that consists of liquids (solutions of various salts in water), gasses (atmospheric as well as water vapour) and solids (mineral particles and organic materials) (Bergström *et al.*, 1998; Yong *et al.*, 1975). It is part of a symbiotic community in which human beings, plants, animals and microorganisms supply in each others needs. Soil could be described as an evolving entity, which is maintained despite continuous changes in the geological, biological, hydrological and meteorological aspects of it (Buol *et al.*, 1977). With the obvious multitude of possible variations in these factors, it is difficult to predict the specific interactions soil would have with external factors. All these factors are closely interdependent and important for the understanding of soil dynamics.

Soil's most important physical properties are its density and the degree of wetness (Yong *et al.*, 1975). Associated with this is water movement, swelling on wetting and cracking on drying, as well as the diffusion of air. The density is dependant on the different particle sizes, their distribution and the size of the voids and pore spaces between them as well as the different minerals and organic matter present. Soil is graded in particle size, from coarse sand (2.0 –0.2 mm), fine sand (0.2 – 0.02 mm), silt (0.02 – 0.002 mm) to clay (<0.002 mm in diameter) (Robinson, 1932). It is important to understand the particle size distributions, because it can influence the chemical, physical and biological properties of the soil.

The larger or granular particles (gravel, sand and silt) form the skeleton of soil and determine its mechanical properties (Yong *et al.*, 1975; Baver *et al.*, 1979). However, the smaller particles with their relatively larger surface area, including colloidal particles (clay), determine most of the physical and chemical properties. There are four colloidal properties that distinguish clay particles from sand and silt (Robinson, 1932). Firstly, clay has a larger ability to retain water by imbibitions than sand or silt. This is similar to the water retention properties of for instance gelatine, agar and silicic acid. Secondly, the water content of colloidal clay notably affects soil volume. This is demonstrated by the cracks that appear in soil when drying. Thirdly, clay has the property of plasticity when associated with certain quantities of water and fourthly, colloidal clay confers certain cohesive properties on soil.

The different soil particles are spaced differently throughout the soil (Robinson, 1932). If all the particles in a theoretical soil were uniformly in size and shape and packed in a closest possible manner, the maximum pore space would be about 26% of the total soil volume. However, the particles in an average soil are not uniform in shape and size. These particles form aggregates with each other and with organic matter, thus the average pore space of most soils is between 40 and 60 % of the total soil volume. It was also found that the composition of the soil particles determine the microbial habitat and hence the microbial community composition in soil (Chenu *et al.*, 2001).

Soil porosity is dependant on soil structure and the size of the aggregates that are present. Soil aggregates improve soil structure and when this takes place the physical properties of the soil, i.e. aeration and water permeability is also improved (Krasil'nikov, 1958; Chenu *et al*., 2000). Structured soil, rich with aggregates, has the ability to absorb and hold water better than unstructured soils. The soil pores are subdivided in two categories, intra-aggregate and inter-aggregate. Intra-aggregate is the pore spaces between the particles inside the soil aggregates. Inter-aggregate is the pore spaces between the various soil aggregates.

The pore space may be filled with water or air (Nielson *et al.*, 1972). Soil air in ideally aerated soils has a relative humidity of up to 98 %, and consists of 78.08% nitrogen, 20.95 oxygen, 0.03% carbon dioxide and 0.94% other gases. However, because of biological activity soil air normally contains much less oxygen and much more carbon dioxide. The composition of soil air usually depends on air movement within the soil system. This occurs as result of fluctuation in soil-water movement like the buoyancy of air bubbles trapped in the soil system. Movement however depends mostly on pore size distribution of the solid matrix.

The portion of the soil volume that is not occupied by solids or gases is occupied by soil water (Nielson *et al*., 1972). Pure water as an entity is very complex, but is even further complicated by interactions with the soil framework. The interactions of water are mostly influenced by three characteristics of the water molecule; its polar nature, its strong tendencies to form hydrogen bonds and its reactive sites that form tetrahedrons. These properties should be considered when trying to understand the interactions of water with soil. However, there are a few properties of water that cannot be understood in its interactions in the soil water system. For instance, the subtle structural changes of water that occurs with changing of temperatures. These structural changes influence the growth of microorganisms and the uptake of potassium by plants.

There are four different types of water that occurs in soil, hygroscopic water, film water, gravitational water/ filtration water and capillary water (Krasil'nikov, 1958). Hygroscopic water is adsorbed directly to the soil particles through molecular cohesion. It has a density of 1, specific heat of 0.9, does not freeze and

can only be moved when transformed into vapour. Film water is basically a second and third layer of water that is loosely bound to soil particles. Gravitational/filtration water moves as liquids and filter down into the soil by gravitational forces. The last water type is capillary water, this type fills the pores between the soil particles and soil aggregates and moves as a result of capillary forces.

Most soil particles are hydrophilic by nature and are therefore able to adsorb water (Nielsen *et al.*, 1972). Different theories are proposed to explain this adsorption. Low (1961) theorized that most of the adsorption is attributed to the hydrogen bonding of water to soils, specifically clays. He proposed that the first layer of water molecules is adsorbed with a hydrogen-bond to the oxygen atoms (and sometimes to the hydroxyl molecules) of clay. This bonding alters the electron distribution of the water molecules, making it easier for them to form hydrogen-bonds with other water molecules. With the repetition of this bonding, a layer of water is formed on the clay surface. This layer is a more orderly structured than ordinary water and has a lower specific volume and greater viscosity and resistance to ionic diffusion than ordinary water.

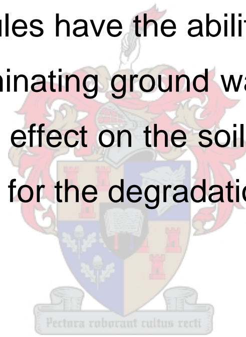
Another theory is that the interfacial attraction of soil for water is largely associated with the hydration and osmotic affects of cations (Bohn *et al.*, 1979). According to this double-layer theory, the electrostatic field of clay particles attracts cations so that some are fixed in the Stern layer (next to the clay surfaces) and beyond the Stern layer the counter ion charge (cation concentration) decreases roughly exponentially with increased distance. Thus, since all this water is carried in a hull or partial hull and an electrostatic field restricts the ions, the water is also restricted. Other forces that may contribute to the adsorption of water by soils are the van der Waal forces and electronic field-water dipole interactions.

Organic matter occurs mainly in the soil as part of plant residues, or as part of manure that was added to the soil as fertilizer (Robinson, 1932; Chenu *et al.*, 2000). It consists partly of recognisable fragments of plant material and material that has lost all recognisable traces of its origin and had became a dark

Pectora roborant cultus recti

amorphous humified matter, better known as humus. Soil rarely contains more than 10 % organic matter, which consists of between 55 and 60 % carbon. Humus also has a colloidal character and shows the same four characteristics as clay, a high capacity to absorb water, the ability to change in volume with wetting and drying, plasticity and cohesion. Humus is also a growth medium for soil microorganisms. These microorganisms decompose the organic matter to form carbon dioxide. For decomposition to take place, certain factors has to be optimised i.e. soil moisture, temperature and available soil air depending on whether the degradation organisms are aerobic or anaerobic.

Another factor that comes into play during modern times, which influences soil degradation processes, is the accumulation of harmful chemicals in soils (Bergström *et al.*, 1998). Chemicals are added to agricultural soil as fertilizers or fungicides (Camobreco *et al.*, 1996). These chemicals can either be mobile or very persistent. Mobile molecules have the ability to move rapidly through soil and may be responsible for contaminating ground water. Persistent molecules remain longer in soil and may have an effect on the soil fertility by negatively effecting soil organisms that are responsible for the degradation of organic compounds.



1.3.2. The characteristics of copper in soil

It was reported that the average Cu concentration in the earth's lithosphere is approximately 70 mg/kg (ppm), but ranges in the top soil from 2 and 100 mg/kg (Baker and Senft, 1995; Lindsay, 1979). However, the concentration of Cu in the earth's crust is more in the region of 24 to 55 mg/kg. It is associated with the organic matter, iron and manganese oxides, soil silicate clays and other naturally occurring minerals (Bååth *et al.*, 1992).

In aerated soil Cu occurs mostly in the divalent cation state namely Cu^{2+} (Knezek *et al.,* 1980; Kabata-Pendias *et al.*, 2001). Between 98.5 and 99.8 % of all soil Cu is in complexes and occurs naturally as sulphides, sulphates, sulphosalts, carbonates and silicates. Clay is able to adsorb Cu above its cation exchange capacity in neutral and alkaline soils (Temminghoff *et al.*, 1997).

Knezek and Ellis (1980) reported that when Cu was in a soil solution, up to 98% of it could form complexes with soil organic matter. Phenolic and carboxylic groups are the most important factors in this adsorption and complex formation. These Cu complexes that form with the soluble and insoluble organic matter are so strong that it may even cause Cu deficiency in some soils (Jenkins *et al.*, 1980). The predominant active Cu ion present in acidic soils (below pH 6.9) is $[Cu(H_2O)_6]^{2+}$, while the predominant Cu ion in neutral to alkaline soils (above pH 6.9) is $Cu(OH)_2^0$ (Baker and Senft, 1995; Ponizovsky *et al.*, 2001).

Cu tends to accumulate in the humus rich surface soil horizon, because of the high adsorption of it to organic matter (McLaren *et al.*, 1973; Wilcke *et al.*, 2002). However, the minerals governing Cu^{2+} solubility is not known but it was shown that Cu solubility is at the minimum between pH 5 and 6 (Lindsay, 1979). The solubility increases as the pH decreases, because of the fewer Cu hydroxyl complexes that are formed, which may be specifically adsorbed. The solubility also increases with an increase in pH, because of the increasing stability of the organo-Cu complexes. Thus, the Cu soil solution concentration depends on (i) Cu-concentration and speciation in the soil solid phase (ii) pH of the soil solution and (iii) the dissolved organic matter concentration in the soil solution (McBride *et al*,. 1997; Temminghoff *et al*., 1994).

Pectora roborant cultus recti

1.3.3. Copper used in agricultural practises

The two most common sources of Cu residues in agricultural soil are fertilizers and fungicides (Loneragan *et al*., 1981; Flores-Véles *et al*., 1996). The average amount of Cu found in vineyards in France is 845 mg/kg and in Germany is 1280 mg/kg. Accumulated concentrations found in plants vary between 1 to 30 mg/kg. In plants, Cu is utilized as a prosthetic group in enzymes, as well as an activator of these systems (Baker and Senft, 1995). Bluestone ($CuSO_4{\cdot}5H_2O$) is the main Cu supplement that's added to the soil as fertilizer (Loneragan *et al*., 1981). This is a highly soluble compound and when in solution, releases a large amount of ions, which can be utilized as nutrients by plants. The oldest known Cu containing fungicide is the Bordeaux mixture (a mixture of $CuSO_4$ and $CaCO_3$), which was

developed in 1878 (Nordgren *et al.,* 1983). On a global scale more than 7×10^7 kg Cu as Bordeaux mixture is being sprayed annually on vines and other crops (Loneragan *et al.,* 1981). Other fungicides that are currently also used include copper oxychloride, copper oxide and copper hydroxide. These fungicides are sprayed on the plant's leafs, and may eventually end up in the soil as part of the organic litter (Loneragan *et al*., 1981).

1.4. SOIL MICROBIOLOGY

In 1902, Oudemans and Koning were the first microbiologists to isolate fungi from soil (Russell, 1923). Although, approximately 45 species of the group *Fungi imperfecti* occurring in soil were identified, it is now estimated that nearly a 1.5 million different species of fungi are present in the environment (Prescott *et al*., 1996; Thorn, 1997). It is believed that there are nearly 5 m of fungal mycelia, 10^8 bacterial cells and 10^6 actinomycete "spores" present in 1 g of soil. The average weight of a bacterial cell or actinomycete "spore" is about 1.5×10^{-12} g and 1 m fungal mycelia is about 9.4×10^{-5} g, making the total weight of the microbial biomass in 1 g of soil approximately 6.0×10^{-4} g. This is about 0.06 % of the total weight of soil.

There are four types of fungi present in soil, motile fungi, Zygomycota, Ascomycetes and Basidiomycetes (Thorn, 1997). Motile fungi include genera related to Protozoa, the chromistan fungi and true fungi. The protozoa like fungi are cellular and plasmodial slime moulds like plant pathogens *Plasmodiophora* and *Spongospora*. The chromistan fungi are oomycetes such as plant pathogens *Pythium* and *Phytophthora*, as well as the hyphochytrids. The true fungi are mostly chytrids, flagellated aquatic fungi that are able move through soil water films. Both Chytrids and hyphochytrids are parasites.

The Zygomycota in soil are symbiotic arbuscular mycorrhizal Glomales, Trichomycetes and Zygomycetes (Thorn, 1997). The Trichomycetes are in symbiosis with insects and the Zygomycetes include genera like *Rhizopus*,

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Mortierella and *Mucor*. The Ascomycetes include yeasts and molds like *Saccharomyces, Lipomyces, Exophiala, Aspergillus, Penicillium, Trichoderma*, and *Fusarium*. The Basidiomycetes present in soil include important lignin degradation fungi, most ectomycorrhizal fungi and some significant plant pathogens. The ligninolytic basidiomycetes include species of the genera *Agaricus, Amanita* and *Coprinus*.

In terms of their biomass, soil fungi are often the dominant microbes in most soils and therefore may represent a significant portion of the nutrient pool (Thorn, 1997). Their activities might even provide or limit access of nutrients to plants. All soil fungi are an intricate part of the complex soil food web either as; food for numerous nematodes, mites, collembolans and tardigrades; as predators and parasites of the above mentioned invertebrates as well as other fauna and microbes; or as recyclers of waste products and other chemicals secreted and excreted by plant roots, animals and microbes.

The main role of microorganisms in soil is that of the decomposition of organic matter (Garett, 1963). Organic matter can be of animal, plant or even microbial origin. Studies conducted during the different stages of decomposition of organic matter in soil, revealed that as the microbial substrate is being depleted, the fungal community composition changes. Three main components of plant material, which are successively utilized by microbes, have been identified. These are sugars and simple carbon compounds, that are easy to decompose and are available to most organisms, and cellulose and lignin which can only be utilize by a minority of organisms. The stages of the decomposition of plant matter are illustrated in Figure 1.1.

Although it was previously accepted that competition between individuals, species, other taxonomic groups and even physiologically related groups, plays a role in shifts observed in microbial community composition during the above mentioned successive stages of decomposition (Garett, 1963), it is now known that other factors may also play a role. Microorganisms that share a common habitat and substrate may maximize their metabolic capabilities through co-operative interactions (Geesy and Costerton, 1986). This may result in the

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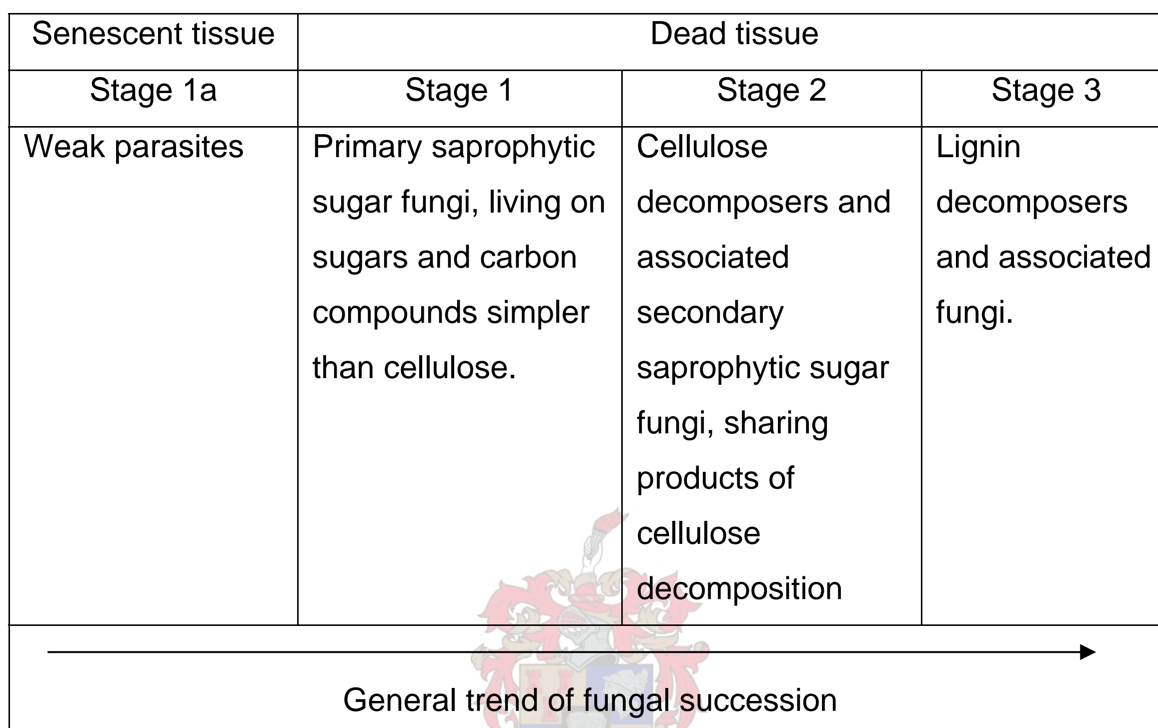
effective utilization of recalcitrant compounds (Wolfaardt *et al.*, 1994). Therefore the fungi observed growing on lignin may not all compete with each other, but may be members of a microbial community in which co-operative interactions occur resulting in the effective decomposition of this substrate.



Senescent tissue	Dead tissue		
Stage 1a	Stage 1	Stage 2	Stage 3
Weak parasites	Primary saprophytic sugar fungi, living on sugars and carbon compounds simpler than cellulose.	Cellulose decomposers and associated secondary saprophytic sugar fungi, sharing products of cellulose decomposition	Lignin decomposers and associated fungi.
→ General trend of fungal succession			

FIG. 1. 1. The three stages of organic matter decomposition. (Garrett, 1963)

1.4.1. Yeast populations present in soil

Although it is known that yeasts commonly occur in soil (Lachance *et al.*, 1998), the extent and composition of many soil yeast communities and their relation to the soil ecosystem are relatively unknown. Most yeasts present in soil are autochthonous and are able to grow in this habitat, because they posses some characteristic adaptive features. Most of these yeasts have a wide spectrum of metabolic abilities, enabling them to aerobically utilize a wide diversity of organic compounds that may end up in soils (Phaff & Starmer, 1987), for example *Lipomyces* species have the unique ability to obtain nitrogen from heterocyclic compounds such as imidazole (La Rue *et al.*, 1967; Van der Walt, 1992).

The capsulated soil yeasts, such as *Cryptococcus*, *Lipomyces* and *Rhodotorula*, are known to survive better in habitats poor in available nutrients (Phaff and Starmer, 1987) such as these prevailing in soil (Williams, 1985). These yeasts were also found to survive periods of desiccation (Phaff and Starmer, 1987). In addition, it was found that a number of soil yeasts species are able to grow under oligotrophic conditions (Kimura *et al*., 1998). These included basidiomycetous species such as *Cryptococcus albidus, Cryptococcus humicolus, Cryptococcus laurentii* and *Rhodotorula glutinis*.

Since it is known that the physicochemical factors of the environment, i.e. energy sources, nutrients, temperature, pH value and water availability, may affect the ecology of yeasts (Do Carmo-Sousa, 1969), and that different yeast species differ in their physiological requirements (Kurtzman and Fell, 1998), it is obvious that the yeast community in soils with different physical and chemical compositions will also differ. However, it was found that most yeasts that occur in soil are found in the upper layer of soil (Pfaff *et al*., 1978).

It was also found that the survival of soil yeasts might depend on other organisms (Pfaff *et al*., 1978). The position of soil yeasts in the organic cycle is to utilize products from the primary attack on plant material carried out by other organisms such as filamentous fungi (Do Carmo-Sousa, 1969). Although it was stated that this is a highly competitive position in this cycle, and that yeasts secrete acidic metabolites to inhibit growth of competitive bacteria, the interactions of soil yeasts with other microbes have not been studied in great detail.

1.4.2. Copper tolerance and resistance in fungi.

Heavy metals such as Cu are used as basis of many fungicides (Wainwright, 1997). Thus, many scientists are interested in the effect these metals have on fungi in the environment. It was found that the relative degree of metal tolerance among soil microflora occurs in the order fungi>bacteria>actinomycetes. Thus, fungi are less susceptible to metal pollution than bacteria (Arnebrant *et al*., 1987). However, the toxicity of a specific metal depends upon the metal species, the organisms that are present and on a variety of environmental factors. It is known

for instance that soil pH may impact on Cu solubility (see par 1.2.2.) and hence the bio-availability of this heavy metal.

Although a range of fungi from all major genera may be found in metal-polluted soil, it is believed that heavy metals affect fungal populations by reducing abundance and species diversity (Wainwright, 1997). It is also found that exposure to heavy metal pollution may select for a more resistant/tolerant fungal population. Thus, even though the total biomass of a contaminated soil may remain unchanged, the structure of the fungal soil community may change (Knight *et al*., 1997). For example, *Geomyces* and *Paecilomyces spp* were found to increase in Cu-polluted soils whereas, *Penicillium* and *Oidodendron spp* decreased (Nordgren *et al*., 1983, 1985). However, some of the best examples of metal tolerance were found within the genus *Penicillium*, for example *Penicillium ochro-chloron* is able to grow in a saturated solution of $CuSO_4$.

Although studies showed that most fungal species isolated from Cu-polluted forest soil were Cu-tolerant (Gadd, 1992, 1993), other authors found that not all strains isolated from Cu-polluted sites were tolerant to this heavy metal, some was even found to be sensitive (Yamamoto *et al.,* 1985). This may be the result of the uneven distribution of Cu in soil, relating to the distribution of the heavy metal among clay minerals, organic matter and soil solution, or even the precipitation of Cu by other microorganisms.

Cu is an important and essential micronutrient to all living organisms (Underwood, 1977), for it has the ability to function as an electron transfer intermediate and it is required as a cofactor for a number of enzymes including copper-zinc superoxide dismutase, cytochrome oxidase (Knight *et al*., 1994) and a copper-metalloenzyme (Gross *et al*., 2000). However, these electron transport capabilities of Cu are also highly detrimental to the cell. Cu may react with the superoxide anion and hydrogen peroxide to form a highly reactive hydroxyl radical via the Fenton reaction, which can attack sugars, amino acids, phospholipids and nucleic acids with disastrous results to the cell (Jensen *et al*., 1998). Cu also has a toxic effect when it binds non-specifically to exposed cysteinyl thiol groups, rendering proteins containing these groups inactive. Thus, it is important for the

cell to regulate Cu homeostasis, from entrance to the final functional destination of the heavy metal.

Fungi have various ways in which to protect themselves against the toxicity of heavy metals (Gadd, 1993; Walker, 1998). The two major strategies are firstly avoidance and secondly sequestration (Tomsett, 1993). Avoidance is when the metal is restricted to enter the cell by either a reduction in uptake/efflux or a formation of a complex outside the cell wall. Sequestration is when the total amount of free ions is reduced in the cytosol of the cell, either by compartmentation of the ions into vacuoles or by synthesis of ligands that can achieve intracellular chelation.

Cu tolerance through avoidance is the first line of defense for fungi (Caesar-Tonthat *et al.,* 1995). Fungi have the ability to secrete compounds that are able to sequester metal ions extracellularly, for instance chelating agents. Some fungi, for instance the brown and white wood rot fungi, have the ability to secrete organic acids like oxalic acid, that are able to form insoluble crystals like copper oxalate, on the outside of the cell wall (Gadd, 1999). Other fungi, such as *Gaeumannomyces graminis* var. *graminis* have the ability to form melanin, a dark pigmented polymer, which can bind Cu (Caesar-Tonthat *et al*., 1995). Chitin and chitosan, polysaccharide cell wall components, are also able to bind Cu and other heavy metals even when the cell is already dead. This phenomenon is called biosorption and is used for the extraction of heavy metals in mining as well as decontamination of polluted sites (Gabriel *et al*., 2001).

Most of the research conducted on the fungal genes involved in Cu resistance was conducted on the yeast *Saccharomyces cerevisiae*. After genetic analysis of Cu sensitive mutants of this species, Welch et al. (1989) identified at least 12 separate complementation groups. The most dominant genes in Cu resistance appeared to be *CUP1* and *ACE1*, as targeted disruption of either gene yields a Cu-sensitive phenotype. However, additional genes were also implicated in Cu homeostasis. The Cu transporter Ctr1 (Dancis *et al*., 1994), homeodomein protein Cup9 (Knight *et al*., 1994), the Cu accumulation protein Bsd2 (Liu *et al*., 1994), the Ace1-like transcription factor Mac1 (Jungmann *et al*., 1993) and the determining

factor for $(CuS)_x$ biomineralization, Slf1 (Yu *et al.*, 1996), were all found to be part of the intricate network involved in Cu homeostasis. However, three main modes of Cu regulation within cells were uncovered. The first is at the point of entry into the cell, the second is regulating cellular Cu through chelation and the third is regulation by partitioning cellular Cu within the cell (Knight *et al.*, 1994).

The main mechanism in yeasts to prevent the cell from being poisoned is the formation of a metallothionein protein that binds intracellular Cu atoms (Fogel, *et al.*, 1983; Oh *et al.*, 1999). The best studied example of this is the metallothionein of *S. cerevisiae* (Butt *et al.*, 1987). The *CUP1* gene, located on chromosome VIII, codes for a cystein rich, low molecular weight protein of approximately 61 amino acids and 10kDa, called Cu metallothionein, copperthionein or copper chelatin. The protein binds less than 10 atoms of Cu with cystein-metal-ion-thiolate bonds forming thiolate bond clusters. The metallothionein (MT) has two main functions, in the presence of high Cu concentrations it protects the cell with the binding process, and in the presence of low physiological concentrations it represses the basal transcription of the *CUP1* promoter (Winge *et al.*, 1985; Wright *et al.*, 1987).

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These *CUP1* genes are non-essential structural genes and disruption and deletion thereof are not lethal, although it causes hypersensitivity towards the heavy metal (Yu *et al.*, 1996). The phenotypic resistant levels to Cu were found to be proportional to the copy number of the gene and may increase and decrease spontaneously by non-reciprocal recombination during mitoses and meioses (Gadd, 1993). Cu sensitive strains usually contain only one copy of the gene but Cu resistant strains carry between 10 and 20 copies and are usually tolerant to 50-100 µM Cu in liquid media (Macreadie *et al.*, 1991; Jeyaprakashet *et al.*, 1991) and to more than 2 mM Cu on solid media (Gadd, 1993).

Cu metallothionein was also studied in various other organisms. The Cu-metallothionein (Cu-MT) gene of *Neurospora crassa* codes for a 26 amino acid protein with a cysteine composition of 28% and it consists of 7 different amino acids (Lerch, 1980). Again all the cysteines are ligated to form Cu complexes with 6 Cu atoms per mol of protein. The Cu binding protein of *Candida albicans* has

also been studied and classified (Oh *et al*., 1999). This Cu-MT binds 7 to 12 atoms of Cu per polypeptide.

The other important Cu resistant gene is *ACE1* also known as *CUP2* (Yu *et al*., 1996; Thiele, 1988). The coding region has several sequence similarities to Cu-MT, including a high number of cysteine residues (Dameron *et al*., 1991). *Ace1* is a trans-acting factor that mediates Cu-induced expression of *CUP1* (Welch *et al*., 1989). Although the *ACE1* gene is constitutively expressed in the absence or presence of Cu, the apoprotein cannot bind DNA. However, it is converted to an active transcription factor upon Cu binding. When the activated *Ace1* forms a tetracopper thiolate cluster (Brown *et al*., 2002), which stabilizes a conformation capable of high-affinity binding to the *CUP1* DNA promoter.

Another well-studied way in which Cu tolerance can be obtained in yeasts, is $(CuS)_x$ biomineralization with the *SLF1* gene as the determining factor (Yu *et al*., 1996). When the external concentration of Cu reaches a certain threshold level, precipitation of CuS occurs on the cell surface. This causes the cell to turn a brownish colour. This CuS forms an exchangeable pool of Cu, while the unexchangeable pool is believed to be situated in the cytoplasm. More than 70% of the Cu associated with the yeast is mineralized in this manner. Disruption in the *SLF1* gene causes a heightened Cu-sensitivity and over-expression results in limited resistance or superresistance towards Cu salts.

Extensive studies have shown that *Mac1*, a nuclear regulatory protein that is related to *Ace1*, is involved in Cu and iron utilization (Jungmann *et al*., 1993). *MAC1* regulates the transcription of *FRE1*, whose function is linked to Cu/Fe reduction. It was found that the MAC1 protein has additional cystein-rich domains that are likely to participate in metal-coordination. The most significant of these is the $CysXCysX_4CysXCysX_2CysX_2His$ motif in the C- terminal region of *MAC1*. These repeats may act as censors for intracellular Cu or Fe concentrations or their redox states. The *MAC1* protein is also able to activate the transcription of *CTR1*, *CTR3* and *FRE7* (a homologue to *FRE1*) under conditions of Cu starvation (Jensen *et al*., 1998). This gene is only expressed in Cu deficient conditions and

is inhibited by a Cu-induced, intramolecular interaction that represses both DNA-binding and transactivation activities (Gross *et al*., 2000).

Another gene found to be important in regulating Cu homeostasis, is *CUP9*. It was hypothesized by Knight *et al* (1994) that this gene is the homeodomein of the Cu homeostasis genes. The homeodomein or homeobox as it is often called, is a region that encodes for a specific binding protein that regulates gene expression by binding to specific sites on the DNA (Zubay, 1993). In the case of *CUP9* it is believed that the gene regulates Cu homeostasis under conditions of active respiration (Knight *et al*., 1994), which happens when the yeast is grown on a non-fermentable carbon sources, for instance lactate. Under these conditions all the energy needs of the cell come from respiration, with a requirement for Cu as co-factor. However, the cell may still be poisoned if the Cu concentration is increased.

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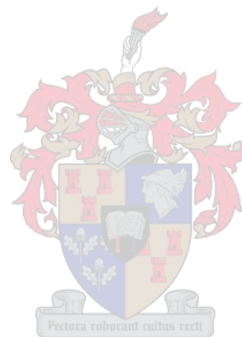


Chapter 2

Determination of copper resistant fungi in soil containing natural low levels of copper.

2.1. INTRODUCTION

The average copper (Cu) concentration in the soil of the earth's crust is about 70 ppm (Baker and Senft 1995). However, in the Western Cape, South Africa, the level of Cu in some soils may be as low as 0.1 ppm (Conradie, 1999). Although some of this Cu may be of natural origin, it was found that soil Cu concentrations might also be affected by agricultural practices (Loneragan *et al.*, 1981). In these cases, Cu reaches the soil as a component of fertilizers and/or fungicides.

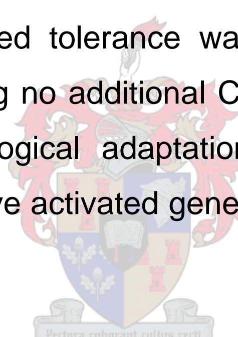
Even though Cu is an important micronutrient of most microbes, it could act as an inhibitor of microbial growth at high concentrations (Gadd, 1993), and may even change the metabolic profile of soil (Duxbury, 1985). Likewise, bacterial numbers in soil were found to drop after Cu application (Bååth *et al.*, 1998). In contrast, filamentous fungi were found to be less susceptible to elevated soil Cu levels than prokaryotes (Arnebrant *et al.*, 1987; Doelman, 1985; Hiroki, 1992). However, relatively little is known about the impact of Cu on the yeast populations in soil.

The number of yeasts in soils may range from a few hundred to more than a million cells per gram (Alexander, 1977; Phaff and Starmer, 1987). These yeasts represent a diverse group of phylogenetically unrelated fungi, many of which are autochthonous (Lachance and Starmer, 1998) and are able to grow in soil by being able to utilize a wide range of organic compounds characteristic of this habitat (Phaff and Starmer, 1987). In addition, a number of soil yeasts are able to grow under oligotrophic conditions (Kimura *et al.*, 1998), which usually prevail in soil (Williams, 1985). Yeast genera commonly encountered in this habitat are *Cryptococcus*, *Lipomyces* and *Rhodotorula* (Phaff and Starmer, 1987; Lachance and Starmer, 1998). While some *Cryptococcus* and *Rhodotorula* strains are known to grow oligotrophically (Kimura *et al.*, 1998), *Lipomyces* species have the rare ability among yeasts to obtain nitrogen by being able to utilize heterocyclic compounds such as imidazole, pyrimidine and pyrazine (LaRue and Spencer, 1967; Van der Walt, 1992). The pyrimidine thymine, was consequently included as nitrogen source in a selective medium to isolate these yeasts from soil (Mothibeli, 1996). Whether other soil yeasts were able to grow on this medium

has never been recorded. If phylogenetically unrelated yeasts were also able to grow on the medium, it could be used as enumeration medium to monitor a larger fraction of the soil yeast community in the presence of elevated soil Cu levels.

Fungi have two major strategies to protect themselves against the toxic effects of Cu. (Tomsett, 1993). The first is avoidance, where Cu is restricted to enter the cell by either reduction of uptake/efflux or complex formation outside the cell. The second strategy is sequestration, where the total amount of free ions is reduced in the cytosol, either by compartmentation of ions into vacuoles, or by synthesis of ligands resulting in intracellular chelation.

It was also found that repetitive culturing of *Cunninghamella blakeslea* and *Rhizopus stolonifer* on agar media containing progressively increasing levels of Cu results in an increased tolerance towards this heavy metal (Garcia-Toledo *et al.*, 1985). However, this acquired tolerance was not stable when cultures were transferred to plates containing no additional Cu. Thus, it was hypothesized that the tolerance was a physiological adaptation rather than an induction of a mutation. The metal might have activated genes coded for biochemical processes that conferred tolerance to Cu.



With the above as background, the aim of this study was to determine whether indigenous fungi of the Western Cape, including yeasts, occurring in soil containing relatively low Cu concentrations, also have the ability to survive and grow in the presence of high Cu concentrations. To achieve this goal the following experiments were conducted:

(1) Soil dilution plates were prepared from different soil samples to determine the proportion of ascomycetous and basidiomycetous yeasts that were able to grow on the selective medium described by Mothibeli (1996). The medium was then used to enumerate a diverse group of unrelated soil yeasts.

(2) A sample of virgin soil, containing a low natural Cu concentration, was subsequently investigated for the presence of fungi able to grow on solid media containing 32 ppm Cu (0.5 mM $CuSO_4$).

(3) To obtain an indication of the level of Cu tolerance of fungi present in this soil sample, fungal isolates were screened for the ability to grow on a series of agar plates containing increasing concentrations of Cu.

(4) Finally, fungi in a series of soil microcosms prepared by experimentally contaminating the virgin soil mentioned above with increasing amounts of Cu, were compared on the basis of plate counts and analysis of the fungal sterol ergosterol in the soil.

2.2. MATERIALS AND METHODS

2.2.1. Estimating the spectrum of yeast taxa able to grow on the selective medium

Soil dilution plates were prepared from four different soil samples using thymine-mineral-vitamin-agar (TMV-agar). This medium was used since it selects for physiologically similar soil yeasts and simultaneously prevents overgrowth of filamentous fungi. The composition of the medium is depicted in Table 2.1. The plates were incubated for one week at 22°C. Subsequently, 20 yeast strains randomly isolated from plates containing between 30 and 300 colonies were selected from each soil sample. Successive inoculation and incubation on malt-extract-agar (MEA) at 22°C were used to purify the isolates. The Diazonium Blue B (DBB) colour reaction (Van der Walt and Yarrow 1984) was used to determine the ascomycetous or basidiomycetous character of each isolate.

2.2.2. Physical and chemical composition of soil investigated for the presence of copper resistant fungi.

The virgin soil that was investigated for the presence of Cu resistant yeasts, originated from Nietvoorbij experimental farm at Stellenbosch, South Africa. It has previously been classified as sandy loam soil of the Clovelly form and the Oatsdale series, derived from quartzite and shale. Approximately 600 kg of virgin soil was collected from an area covered with pristine vegetation. The soil was subsequently dried for two weeks at 30°C and sieved (2000 μm grid). The main

chemical and physical properties of the soil were determined and are listed in Table 2.2.

2.2.3. Investigating virgin soil for the presence of copper resistant fungi.

Cu resistant fungi in the soil were enumerated using two different plating techniques and six different isolation media, each containing 32 ppm Cu (0.5 mM $CuSO_4$). This concentration of Cu is known to select for Cu resistant fungi (Thiele, 1988).

2.2.3.1. Enumeration of fungi using the dilution plate technique. Five replicate series of soil dilutions were prepared by using autoclaved physiological salt solution (0.89% w/w NaCl) containing 0.1% agar (PSA). To enumerate a wide diversity of filamentous fungi and yeasts, the 1/10, 1/100, 1/1000, 1/5000 and 1/10000 dilutions of each series of dilutions were used to inoculate malt extract agar (MEA), malt extract agar with streptomycin sulfate (MEAs), as well as malt-yeast–extract-peptone agar with chloramphenicol and streptomycin sulfate (MYPps) (Carreiro and Koske, 1992). The compositions of these media, containing 32 ppm additional Cu, are listed in Table 2.3. Hundred microlitre aliquots of each soil dilution were used to prepare spread plates of the above mentioned media.

TMV-agar (Table 2.1) containing 32 ppm additional Cu was used to enumerate Cu resistant lipomycetaceous and basidiomycetous soil yeasts. Hundred microlitre aliquots of the 1/10, 1/100 and 1/1000 dilutions in each of the five series of soil dilutions mentioned above were used as inocula for spread plates prepared with TMV-agar.

A modification of the benomyl–dichloran-streptomycin medium (BDS-medium) of Worrall (1991) was used to enumerate Cu resistant hymenomycetous fungi in the soil (Table 2.4). Hundred microlitre aliquots of the 1/10, 1/100, 1/1000, 1/5000, and 1/10000 dilutions in each of the five series of soil dilutions were used to inoculate spread plates prepared with BDS-medium.

Table 2.1. The composition of thymine-mineral-vitamin (TMV) medium used to enumerate lipomycetaceous and basidiomycetous yeasts present in soil (Adapted from Mothibeli, 1996).

Components per litre of distilled water.			
Carbon source		*Vitamins*	
Glucose (g)	5.00	Biotin (μg)	1.00
Nitrogen source		Calcium pantothenate (μg)	200.00
Thymine (g)	0.10	Folic acid (μg)	1.00
Mineral salts		Inositol (μg)	1000.00
$CaCl_2$ (g)	0.10	ρ- aminobenzoic acid (μg)	100.00
KH_2PO_4 (g)	1.00	Pyridoxine hydrochloride (μg)	200.00
$MgSO_4{\cdot}7H_2O$ (g)	0.50	Riboflavin (μg)	100.00
NaCl (g)	0.10	Thymine (μg)	500.00
Trace elements		*Copper supplement*	
$AlK(SO_4)_2{\cdot}12H_2O$ (μg)	10.00	$CuSO_4{\cdot}5H_2O$ (mg)	124.90
$CoSO_4$ (μg)	100.00	*Anti-bacterial agent*	
$FeCl_3{\cdot}6H_2O$ (μg)	200.00	Chloramphenicol (g)	0.20
H_3BO_3 (μg)	500.00		
KI (μg)	100.00	*Solidifying agent*	
$MnSO_4{\cdot}H_2O$ (μg)	40.00	Agar (g)	10.00
$Na_2MoO_4{\cdot}H_2O$ (μg)	200.00		
$ZnSO_4{\cdot}7H_2O$ (μg)	400.00	*Final pH*	5.20

After inoculation, all the above mentioned plates were incubated for 7 days at 22°C, and fungi growing on the plates were enumerated as colony forming units (cfu). Five replicate plates of each dilution were prepared with each of the above mentioned media. Controls containing no additional Cu were included in all of the experiments.

Table 2.2. Characteristics of the virgin soil taken at the sampling site on Nietvoorbij experimental farm.

Physical Characteristics	
[1]*Texture*	
Stone (%) (Particle diameter >2.0 mm)	3.00
Coarse Sand (%) (Particle diameter 0.5 – 2.0 mm)	30.90
Medium Sand (%) (Particle diameter 0.2 – 0.5 mm)	13.10
Fine Sand (%) (Particle diameter 0.02 – 0.2 mm)	24.50
Silt (%) (Particle diameter 0.002 – 0.02 mm)	22.80
Clay (%) (Particle diameter < 0.002 mm)	5.70
Chemical Characteristics	
[2]Organic Carbon (%)	3.54
[3]Total Nitrogen (%)	0.20
[4]Ammonium (ppm)	5.40
[5]Nitrate and Nitrite (ppm)	3.20
Potassium (ppm)	245.00
[6]Phosphorous (ppm)	29.00
[7]Copper (ppm)	1.89
[8]Zinc (ppm)	6.80
[9]Manganese (ppm)	80.00
[10]Boron (ppm)	0.86
[11]Exchangeable cations	
Calcium (cmol/kg)	4.70
Potassium (cmol/kg)	0.63
Sodium (cmol/kg)	0.11
Magnesium (cmol/kg)	3.25
[12]pH of a soil suspension containing 1 part soil and 2.5 parts 1 M KCl.	6.10

[1]Determined by Bemlab CC using the hydrometer method (Van der Watt, 1966)

[2]Determined by Bemlab CC using the Walkley–Black method (Nelson and Sommers, 1982)

[3]Determined by Bemlab CC through digestion in a LECO FP-528 nitrogen analyser.

[4-5]Determined in a 1M KCl extract by Bemlab CC (Bremner, 1965).

[6]Determined in a Bray – 2 extract by Bemlab CC (Thomas and Peaslee, 1973).

[7-9]Determined in a di-ammonium EDTA extract by Bemlab CC according to the methods of Beyers and Coetzer (1971).

[10]Determined in a hot water extract by Bemlab CC according to the methods of the Fertilizer Society of South Africa (1974).

[11]Determined in a 1 M ammonium acetate extract by Bemlab CC according to the methods of Doll and Lucas (1973)

[12]Determined by Bemlab CC according to the method of McClean (1982)

Table 2.3 The compositions of relative non-selective fungal isolation media that were used to enumerate filamentous fungi.

Components	MEA	MEAs	[1]MYPps
Components per liter of distilled water			
Carbon source			
Malt extract (g)	20.00	20.00	7.00
Peptone (g)			1.00
Yeast extract (g)			0.50
Anti-bacterial agents			
Streptomycin sulfate (g)		0.50	0.50
Chloramphenicol (g)			0.50
Copper supplement			
$CuSO_4 \cdot 5H_2O$ (mg)	124.90	124.90	124.90
Solidifying agent			
Agar (g)	20.00	20.00	20.00

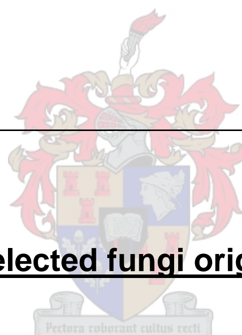
[1]Prepared according to the method of Carreiro and Koske (1992).

2.2.3.2. Enumeration of mucoralean fungi using the soil plate technique. Recently, the soil plate technique was successfully used to enumerate mucoralean fungi in different soils (Strauss *et al*., 2000). It was therefore decided to include this plating method in the experimentation. Soil plates, each inoculated with 0.001 g soil, were prepared according to the methods described by Strauss *et al*., (2000). Five replicate plates prepared with *Mucor* isolation medium A (MucA), as well as five replicate plates prepared with *Mucor* isolation medium B (MucB), each containing 32 ppm Cu, were used to enumerate the mucoralean fungi (compositions of media are depicted in Table 2.5). Inoculated agar plates were incubated for 7 days at 22°C after which the fungal cfu on the plates were determined. Controls containing no additional Cu in the medium were included in all of the experiments.

Table 2.4. Composition of benomyl–dichloran–streptomycin medium (BDS-medium) used for the enumeration of hymenomycetous fungi present in the soil (Adapted from Worrall, 1991).

Components per liter of distilled water	
Carbon source	
Malt extract (g)	15.00
Anti-fungal agents	
Benomyl (mg)	2.00
[1]Dichloran (mg)	2.00
Phenol (mg)	50.00
Anti bacterial agent	
Streptomycin sulfate (mg)	100.00
Copper supplement	
$CuSO_4{\cdot}5H_2O$ (mg)	124.90
Solidifying agent	
Agar (g)	15.00

[1]2,6-dichloro-4-nitroaniline



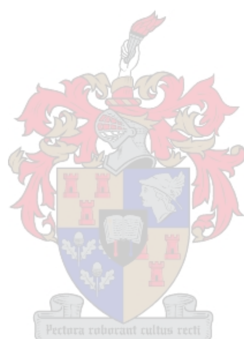
2.2.4. Copper tolerance of selected fungi originating from the virgin soil

Forty fungal strains obtained from the virgin soil sample (Par. 2.2.2.), were randomly isolated at 22°C on MEA. These isolates, including filamentous fungi and yeasts, were identified using morphological and molecular criteria.

2.2.4.1. Identification of filamentous fungi. Fungi grown on MEA-medium at 22°C were identified according to the keys of Domsch *et al.,* (1980).

Table 2.5. The compositions of selective isolation media for the enumeration of mucoralean fungi that were used in the experiment (Strauss *et al*., 2000).

Components	MucA	MucB
	Components per litre of distilled water	
Carbon sources		
Starch (g)	10.00	
Sucrose (g)		10.00
Yeast extract (g)	0.50	0.50
Mineral salts		
NH_4Cl (g)	1.00	1.00
KH_2PO_4 (g)	1.00	1.00
$MgSO_4 \cdot 7H_2O$ (g)	0.50	0.50
$FeSO_4 \cdot 7H_2O$ (mg)	10.00	10.00
$ZnSO_4 \cdot 7H_2O$ (mg)	10.00	10.00
$MnSO_4 \cdot H_2O$ (mg)	0.80	0.80
$CuSO_4 \cdot 5H_2O$ (mg)	0.05	0.05
Copper supplement		
$CuSO_4 \cdot 5H_2O$ (mg)	124.90	124.90
Anti bacterial agent		
Chloramphenicol (g)	0.20	0.20
Anti-fungal agent		
[1]Benomyl (g)	0.02	0.02
Solidifying agent		
Agar (g)	16.00	16.00
Final pH	5.50	5.50



[1]Aldrich catalog no. 38,158-6

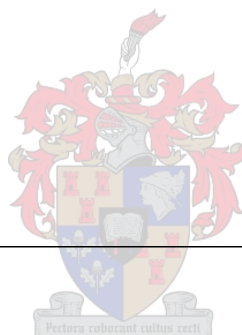
2.2.4.2. Identification of yeast isolates. Yeast isolates were grown for 24 h in 20 ml of yeast-peptone-dextrose (YPD) broth (2% glucose, 2% peptone, 1% yeast-extract). Genomic DNA was extracted according to the method of Hoffman and Winston (1987). The D1/D2 600-650bp region of the large subunit of ribosomal DNA (rDNA) was subsequently amplified using the polymerase chain reaction (PCR). The DNA was amplified with the forward primer F63 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and the reverse primer LR3 (5'-GGT CCG TGT TTC

AAG ACG G-3') in a Perkin-Elmer thermal cycler (Fell *et al.*, 2000). The PCR products were purified with Nucleospin® (Separations) chromatography columns. Sequences representing the D1/D2 region of the rDNA from the strains were obtained using an ABI PRISM model 3100 genetic sequencer. The forward and reverse sequences were aligned with DNAMAN for WINDOWS Version 4.13 (Lynnon Biosoft). The yeast strains were identified by comparing the sequencing results with known sequencing results using the BLAST program (www.ncbi.nlm.nih.gov/blast).

2.2.4.3. Determination of copper tolerance. The fungal isolates mentioned above, including filamentous fungi and yeasts, were each inoculated onto a series of plates containing glucose-glutamate-yeast extract as basal medium, with different concentrations of $CuCl_2 \cdot 2H_2O$ added to it (Mergeay, 1995). The composition of the basal medium is depicted in Table 2.6. and the final Cu concentrations were 1, 10, 50 and 100 ppm. Inoculation of filamentous fungi was performed by cutting equal sized squares (2 mm x 2 mm) from two-week-old cultures on MEA plates and aseptically transferring each to the center of a Cu containing plate. Radial growth of each filamentous fungus was recorded periodically up to 46 days, depending on the growth rate of the fungus. Screening yeasts for their ability to grow on the above mentioned Cu containing medium was conducted by inoculating the medium with growth from a two-week-old culture on MEA and incubating the plate at 22°C for 3 days. The presence or absence of growth on the plates was subsequently recorded.

Table 2.6. Composition of the glucose-glutamate-yeast extract basal medium used to determine growth of selected fungal cultures in the presence of elevated Cu levels (Mergeay, 1995).

Components per litre of distilled water	
Carbon sources	
Glucose (g)	10.00
Glutamic acid (g)	1.00
Yeast extract (g)	0.10
Mineral salts	
NaCl (g)	0.20
K_2HPO_4 (g)	0.50
$MgSO_4$ (g)	0.20
Vitamins	
Biotin (μg)	1.00
Thiamine (μg)	10.00
Solidifying agent	
Agar (g)	20.00
Final pH	6.00



<u>2.2.5. Impact of increased copper concentrations on soil fungal populations.</u>

2.2.5.1. Preparation of soil microcosms. A series of soil microcosms was prepared by adding copper oxychloride ($CuCl_2 3Cu(OH)_2$) to 1.5 kg aliquots of virgin soil, contained in plastic soil bags, in the following concentrations: 0, 10, 20, 30, 40, 50, 100, 500, and 1000 ppm. The resulting concentration of Cu in a di-ammonium EDTA extract of the soil in each microcosm was subsequently determined and is depicted in Table 2.7. Each microcosm containing a different Cu concentration was prepared in triplicate. The experiment was initiated by adding distilled water to each microcosm (final moisture content, *circa* 15% v/w). Thereafter, 200 ml water was added every two weeks to each microcosm. The soil was incubated at 22°C and analysed using the methods described below.

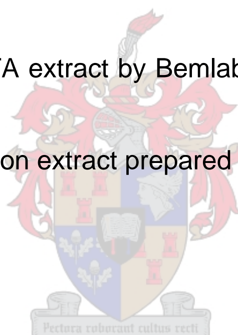
Table 2.7. The final Cu concentrations[1] in the series of soil microcosms used in the experimentation.

	Cu (mg/kg)[2]	Cu (mg/l)[3]	% saturation
Control	1.89 (0.04)	0.0043 (0.0015)	46.8 (0.9)
10 ppm	12.18 (0.88)	0.026 (0.0014)	46.4 (1.5)
20 ppm	22.61 (0.70)	0.0357 (0.0025)	45.7 (1.1)
30 ppm	33.79 (4.50)	0.0453 (0.0046)	45.2 (0.4)
40 ppm	42.14 (3.35)	0.056 (0.0026)	43.8 (0.3)
50 ppm	59.46 (3.38)	0.0673 (0.0060)	43.9 (1.2)
100 ppm	125.69 (2.90)	0.1047 (0.0038)	47.4 (1.2)
500 ppm	516.12 (6.70)	0.3527 (0.0213)	43.9 (1.1)
1000 ppm	1112.40 (52.17)	0.947 (0.0719)	49.8 (2.3)

[1] The values are the means of three repetitions while the values in brackets represent the standard deviation of each mean.

[2] Determined in a di-ammonium EDTA extract by Bemlab CC according to the methods of Beyers and Coetzer (1971).

[3] Determined in a saturated soil solution extract prepared according to the methods of Longenecker and Lyerly (1964).



2.2.5.2. Enumeration of soil fungi. Soil was collected periodically (after 0, 2, 7, 14, 28, 70 and 245 days of incubation) to enumerate the different fungal populations. To enumerate fungi representing a wide diversity of taxa, a 1/1000 soil dilution was used as inoculum for MEAs. A 1/100 soil dilution was used to inoculate BDS-medium to enumerate hymenomycetous fungi. Soil yeasts were enumerated using a 1/10 soil dilution as inoculum for TMV-medium. The mucoralean fungi were enumerated by using the soil plate technique in combination with MucA medium. Additional plates containing 32 ppm Cu were used to determine the number of Cu resistant fungi, able to grow on each of the above mentioned media. These Cu resistant fungi were enumerated after 0, 14, 70 and 245 days of incubation of the soil samples.

2.2.5.3. Determination of fungal biomass using sterol analyses of soil. After 245 days of incubation soil samples were taken from the microcosms that received

0, 50, 100 and 1000 ppm Cu. The sterols in each of these soil samples were hydrolysed and extracted according to the method of Eash *et al*. (1996). Soil (5 g), 15 ml cold (0°C) methanol and 5 ml of alkaline solution (40 g/l KOH in 95 % ethanol) were mixed in a centrifuge tube, using a vortex mixer and a sonicator. The extraction mixture was subsequently transferred to a pressure reaction tube and heated at 85°C for a total of 30 min. However, after 15 min the tube was temporary removed for 1 min to vortex the contents. After heating, each tube was allowed to cool and 5 ml deionised water was added.

The extraction mixture in each tube was filtered and washed with methanol (Eash *et al*., 1996). The filtrate was transferred to a 250 ml separatory funnel and extracted three times with pentane (3 x 10 ml). The pentane layer was transferred to an evaporation tube after which the tube was placed in an evaporator (60°C) and dried under N_2 gas. Each sample was dissolved in 5 ml methanol and homogenized using a vortex mixer. The suspension was subsequently filtered and high performance liquid chromatography (HPLC) was performed using a Phenomenex Luna 5 μ C18(2) column (25 cm x 0.46 cm, diameter) in a Beckman System Gold Diode Array HPLC system. A mixture of methanol: deionised water (99.50: 0.50, by vol.) was used as solvent phase at a flow rate of 1.2 ml/min. Detection was carried out simultaneously at 210 and 280 nm.

Peaks representing sterols were identified using authentic standards. In addition, concentrations of sterols in the sample were calculated using the ratio of the peak area in the sample chromatogram to the sterol peak area in a chromatogram of a standard solution. The standard stock solution (prepared with ethanol) contained 2.53 μg/10 μl ergosterol, 2.53 μg/10 μl lanosterol, 2.50 μg/10 μl stigmasterol and 2.72 μg/10 μl squalene.

2.3. RESULTS AND DISCUSSION

2.3.1. Estimating the spectrum of yeast taxa able to grow on the selective medium.

Using the DBB test on 80 yeast isolates randomly selected from soil dilution plates, prepared from four different soil types, it was found that 94.4% were basidiomycetes while 5.6% were ascomycetous yeasts. Phylogenetically unrelated yeast taxa were therefore able to grow on the medium and it was decided to use the medium as an enumeration medium for unrelated but physiologically similar soil yeasts.

2.3.2. Investigating virgin soil for the presence of copper resistant fungi.

The results obtained when Cu resistant fungi in virgin soil were enumerated on different isolation media, each containing 32 ppm Cu, are illustrated in Figures 2.1 to 2.6. Interestingly, significant differences in microbial numbers were observed when MEA with 32 ppm Cu and MEA without additional Cu were used as enumeration medium (Fig 2.1). This medium was the least selective of the enumeration media and contained no anti-bacterial agents. Consequently, differences mentioned above may be ascribed to Cu-sensitive bacteria in the soil, since no such differences were observed on dilution plates prepared from MEA containing streptomycin sulfate (MEAs) as anti-bacterial agent (Fig 2.2). No significant differences regarding the microbial numbers in the presence of 32 ppm Cu and in the absence of additional Cu were observed on soil plates prepared with MYPps, BDS, as well as with MucA and MucB (Fig 2.3, 2.4 and 2.6). Cu resistant fungi, including hymenomycetous and mucoralean fungi, therefore did exist in virgin soil containing less than 2 ppm Cu (Table 2.1).

However, yeasts in the soil were found to be sensitive for elevated Cu levels (Fig 2.5). Significantly less of these unicellular fungi were able to grow on TMV-medium containing 32 ppm Cu, than on the TMV-medium without the additional Cu.

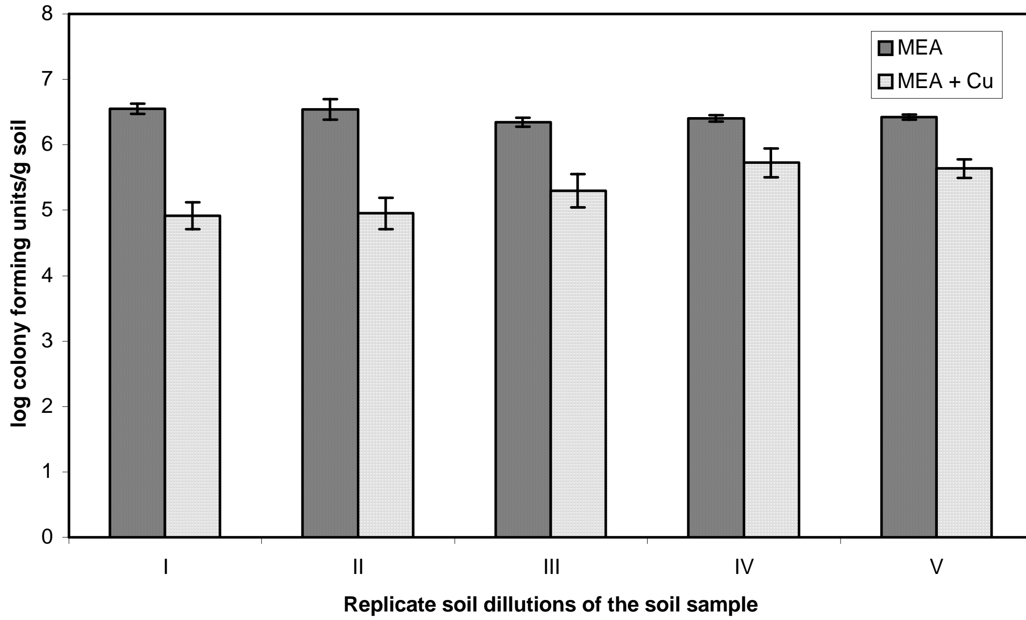


FIG 2.1. The number of microorganisms able to grow on malt extract agar (MEA) and malt extract agar containing 32 ppm additional copper (MEA + Cu)

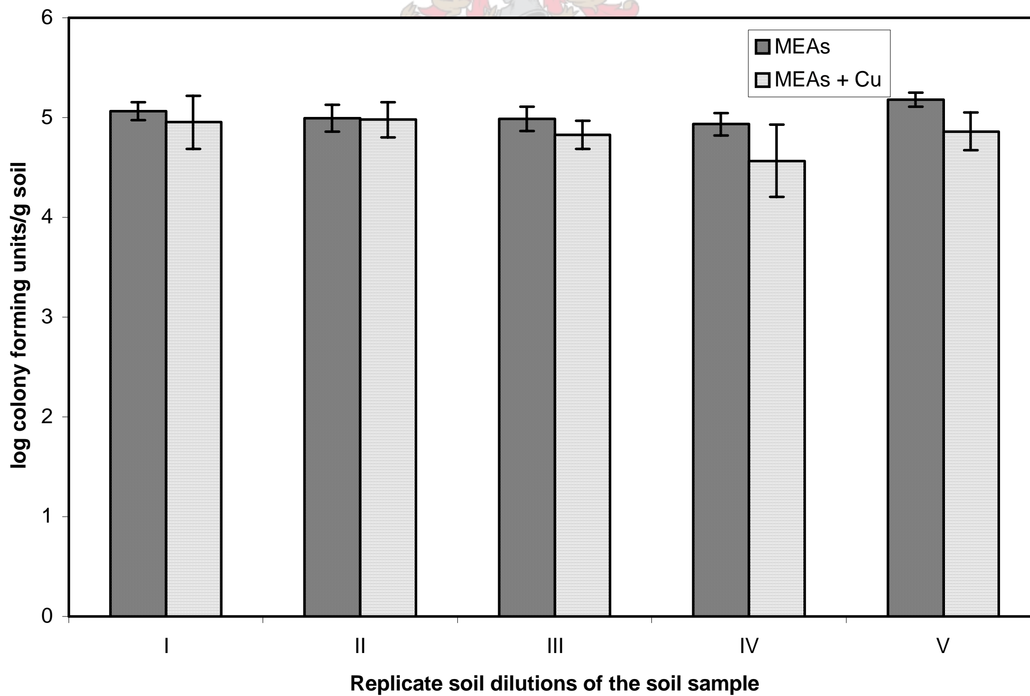


FIG 2.2. The number of microorganisms able to grow on malt extract agar containing streptomycin sulfate (MEAs) and malt extract agar containing streptomycin sulfate and 32 ppm additional copper (MEAs + CU)

* The standard deviation is indicated on top of each bar which in turn represents the mean of five repetitive counts.

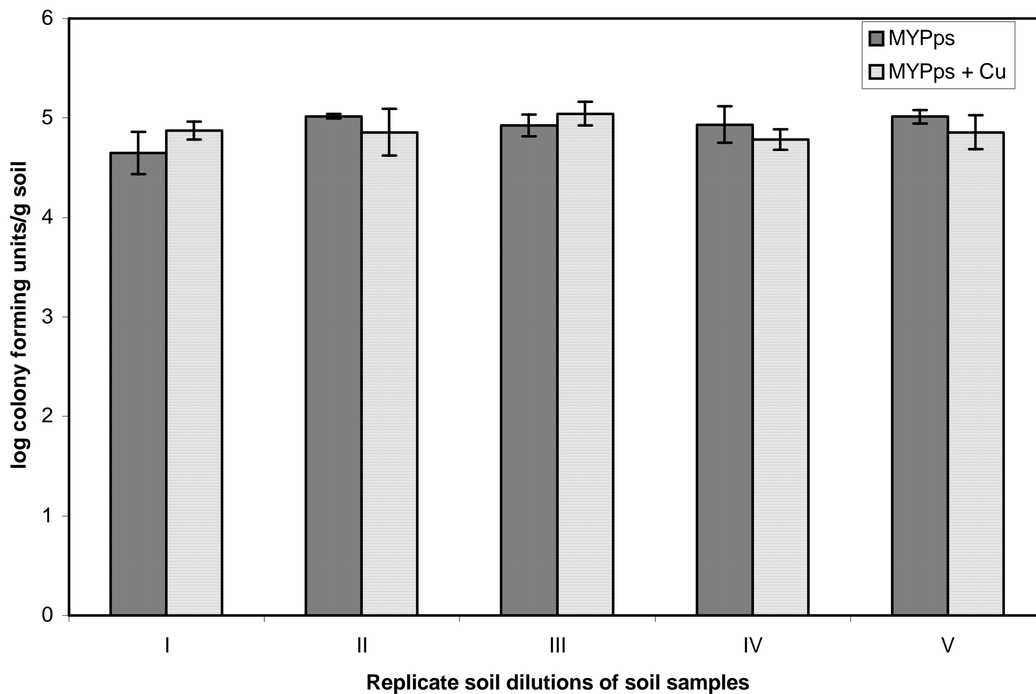


***FIG 2.3**. The number of microorganisms able to grow on malt-yeast-extract-peptone agar (MYPps) and malt-yeast-extract-peptone agar containing 32 ppm additional copper (MYPps + Cu)

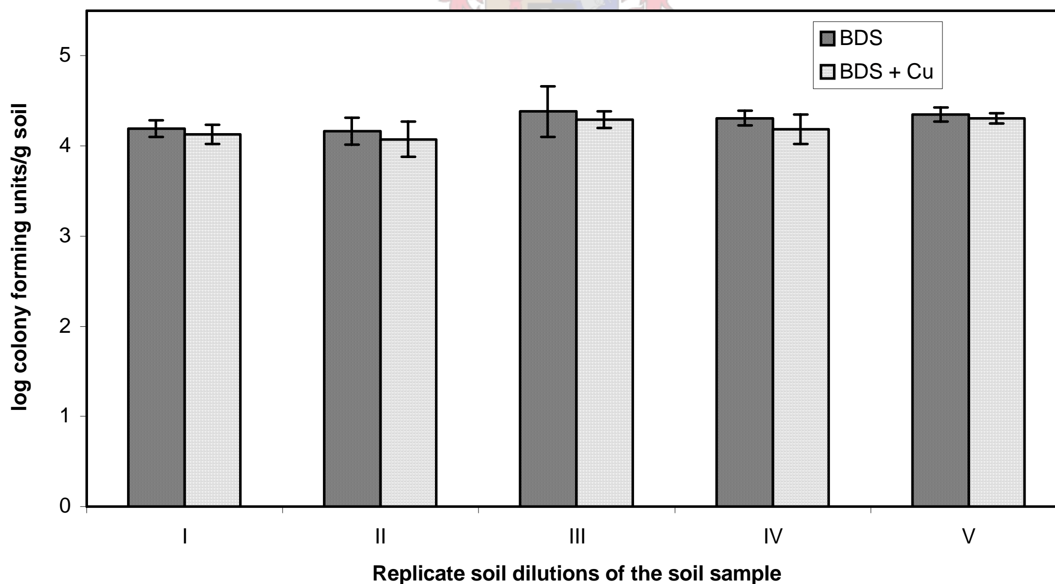


***FIG 2.4.** The number of hymenomycetous fungi able to grow on benomyl-dichloran-streptomycin agar (BDS) and benomyl-dichloran-streptomycin agar containg 32 ppm additional copper (BDS + Cu).

* The standard deviation is indicated on top of each bar that in turn represents the mean of five repetitive counts.

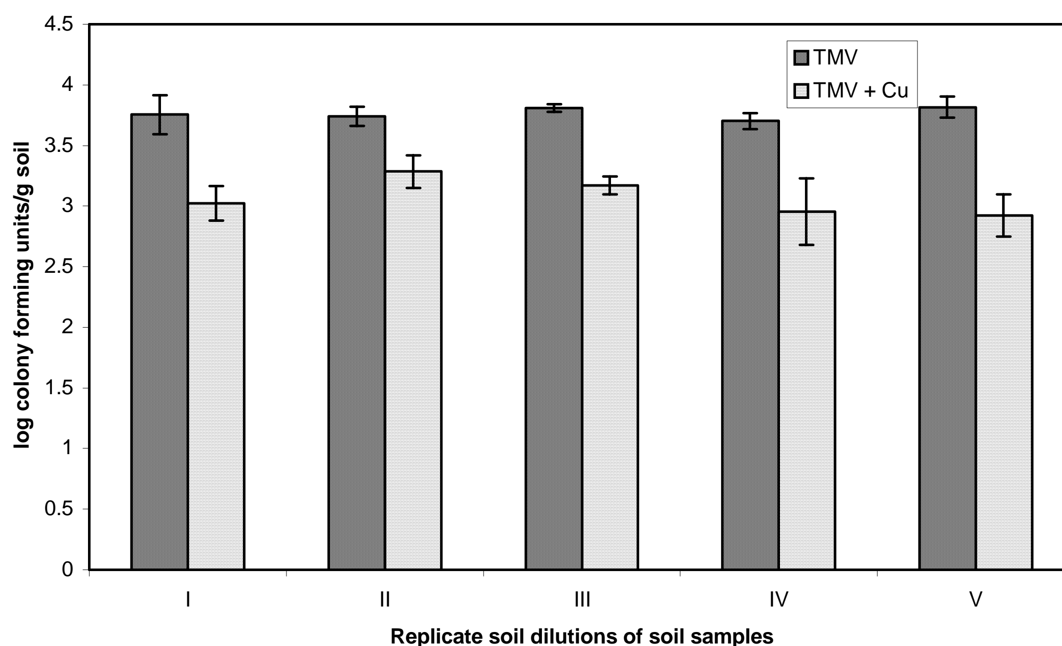


***FIG 2.5**. The number of soil yeasts able to grow on thymine-mineral-vitamine agar (TMV) and thymine-mineral-vitamin agar containing 32 ppm additional copper (YMV + Cu)

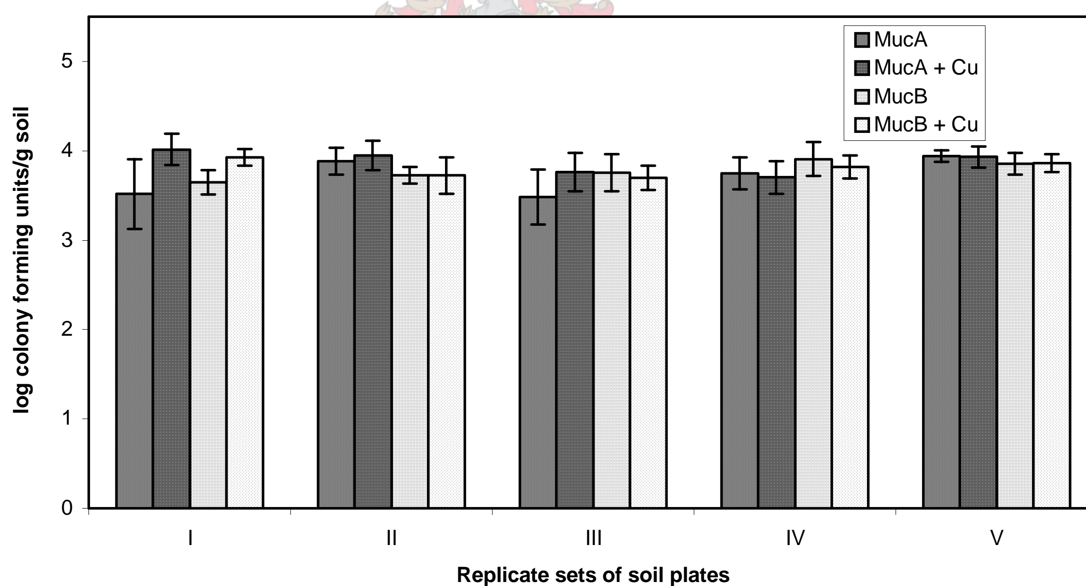


***FIG 2.6**. The number of mucoralean fungi able to grow on soil plates prepared with Mucor isolation media A and B (MucA and MucB) and MucA and Mucb containing 32 ppm additional copper (MucA + Cu, and MucB + Cu)

* The standard deviation is indicated on top of each bar that in turn represents the mean of five repetitive counts.

2.3.3. Copper tolerance of fungi isolated from virgin soil.

To obtain an indication of the level of Cu tolerance of fungi occurring in the soil, randomly isolated strains were challenged with increasing amounts of Cu in a growth medium. Radial growth in the presence of different Cu concentrations was determined for the filamentous isolates (Table 2.8), while yeasts were screened for growth using the same series of Cu concentrations (Table 2.9). All the isolates were able to grow in the presence of 10 ppm Cu and despite a natural Cu concentration of less than 2 ppm (Table 2.1) most of the isolates were able to grow in the presence of up to 50 ppm Cu. However, most of the isolates were unable to grow on the medium containing a 100 ppm added Cu.

Evidence for Cu as micronutrient of filamentous fungi was obtained, since radial growth of lower fungi belonging to the Mucorales, Mortierellales and Oomycota (i.e. *Absidia, Mortierella, Pythium* and *Rhizopus*), as well as most of the *Penicillium* and Hymenomycetous strains, was stimulated at 10 ppm Cu in the medium (Table 2.8). Similar results were obtained by Cuero *et al.,* 2003 when *Aspergillus flavus* was challenged with 5mg/L Cu. The *Penicillium* isolates used in the experiment were generally not able to grow on the medium containing 100 ppm Cu. The *Micromucor* isolates were all able to grow on the medium containing 100 ppm Cu. Differences in the ability to grow at 100 ppm Cu also existed among the yeasts isolated from the virgin soil, even between strains of the same species (Table 2.9). Most of the *Cryptococcus podzolicus* strains and the *Cryptococcus* species 2 strains were able to grow in the presence of 100 ppm Cu. The *Cryptococcus* species 1 strain CBS 8372, the strain representing *Cryptococcus phenolicus* and the strains originating from other habitats were unable to grow in the presence of 100 ppm Cu.

2.3.4. Impact of increased copper concentrations on soil fungal populations.

It is known that Cu is a relatively immobile element in soil and is readily adsorbed to soil organic compounds (Loneragan *et al*., 1981). Thus, when increasing amounts of copper oxychloride were added to a series of soil microcosms, the level of Cu in the soil solution was notably less than the level of

bio-available Cu as determined in the EDTA-extract, and the total concentration of added Cu in each microcosm (Table 2.7). It could therefore be assumed that when Cu was added to the soil, only the fraction in the soil solution extract would be in direct contact with fungi in the soil.

When the fungal colony forming units (cfu's) were monitored in the different soil microcosms, it was found that fungal numbers changed over time (Figs. 2.7, 2.8, 2.9, and 2.10). For example, the number of cfu's representing filamentous fungi, obtained on MEAs, generally seemed to peak after 2 to 14 days in the soil microcosms (Fig 2.7). However, compared to the control microcosms containing no additional Cu, the number of filamentous fungi, generally showed no significant change with the addition of Cu to the microcosms. Similar results were obtained when the hymenomycetous fungi were enumerated in the microcosms (Fig 2.8). The results support the findings that Cu resistant filamentous fungi, including the hymenomycetous fungi, exist in soil. It must however be noted that the number of filamentous fungi, enumerated using the abovementioned techniques, did not only represent fungal biomass, but also the extent of sporulation (Russell, 1923).

The results obtained when the numbers of mucoralean fungi were monitored using the soil plate technique (Fig 2.9), indicated that Cu resistant mucoralean fungi existed in virgin soil. Generally, no notable differences were observed between the control microcosms and those that received Cu. However, after 14 days of incubation, more mucoralean fungi were observed in the microcosms that contained 1112 ppm Cu than in the control microcosms. It must be noted that the soil plate technique selects for fungi that do not sporulate abundantly (Warcup, 1950) and that many of the colonies observed on these plates may be as a result of germinating hyphal fragments. Therefore, the increase in mucoralean numbers might have been as a result of an increase in the biomass of Cu resistant fungi in the absence Cu resistant competitors and/or predators.

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Table 2.8. Radial growth calculated as a percentage of the maximum colony radius that was measured after the indicated period of incubation[1].

Culture	Days	0 ppm	1 ppm	10 ppm	50 ppm	100 ppm
Absidia R1	8	98	100	100	40	0
Absidia R2	8	90	94	100	70	0
Absidia R3	7	99	100	100	67	0
Absidia R31	8	91	94	100	93	45
Fusarium R14	10	100	100	100	62	0
Hymenomycete R21	34	94	100	95	0	0
Hymenomycete R24	46	85	93	100	0	0
Hymenomycete R26	29	91	100	78	72	79
Micromucor R22	46	97	93	100	92	27
Micromucor R25	46	92	100	82	78	65
Micromucor R28	46	95	93	100	87	60
Mortierella R5	14	95	94	100	100	0
Mortierella R29	15	95	93	100	50	0
Mortierella R30	14	96	99	100	45	1
Penicillium R4	46	86	96	100	96	0
Penicillium R6	46	68	96	100	11	0
Penicillium R7	15	89	100	87	74	7
Penicillium R8	46	100	83	79	74	48
Penicillium R9	14	99	100	87	28	0
Penicillium R10	46	86	87	100	92	7
Penicillium R13	13	100	96	90	49	16
Penicillium R15	46	89	100	99	57	0
Penicillium R16	46	86	100	76	31	0
Penicillium R17	15	99	100	89	67	0
Penicillium R19	46	80	94	100	92	0
Penicillium R20	46	95	100	100	67	10
Pythium R12	46	95	85	100	17	0
Rhizopus R32	3	99	100	100	62	0
Trichoderma R11	4	100	100	100	45	0

[1]Values are the means of three repetitions. Standard deviations were all less than 10%.

Fungal strains were identified according to the keys of Domsch *et al.* (1980)

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Table 2.9. The ability of yeast[1] isolates to grow on plates containing different Cu concentrations[2].

Culture	0 ppm	1 ppm	10 ppm	50 ppm	100 ppm
C. phenolicus R35	+	+	+	+	-
C. podzolicus R34	+	+	+	+	+
C. podzolicus R36	+	+	+	+	+
C. podzolicus R37	+	+	+	+	+
C. podzolicus R40	+	+	+	+	-
C. podzolicus R41	+	+	+	+	+
[3]*Cryptococcus* sp. 1 R33	+	+	+	+	-
[4]*Cryptococcus* sp. 2 R38	+	+	+	+	+
[4]*Cryptococcus* sp. 2 R39	+	+	+	+	-
[4]*Cryptococcus* sp. 2 R42	+	+	+	+	+
[5]*Lipomyces starkeyi* R45	+	+	+	+	-
[5]*Rhodotorula graminis* R44	+	+	+	+	-
[5]*Saccharomyces cerevisiae* R43	+	+	+	+	-
[5]*Schwanniomyces occidentalis* R46	+	+	+	+	-

[1]Yeast isolates identified according to the D1/D2 region method of Fell *et al.* (2000).

[2]Key: + indicates growth after 3 days of incubation; - indicates no growth after 3 days of incubation.

[3]*Cryptococcus* sp resembling *Cryptococcus* sp culture CBS 8372.

[4]*Cryptococcus* sp resembling *Cryptococcus* sp culture KCTC 17078.

[5]Yeast strains originating from other habitats were included to test a wider spectrum of phylogenetically diverse taxa.

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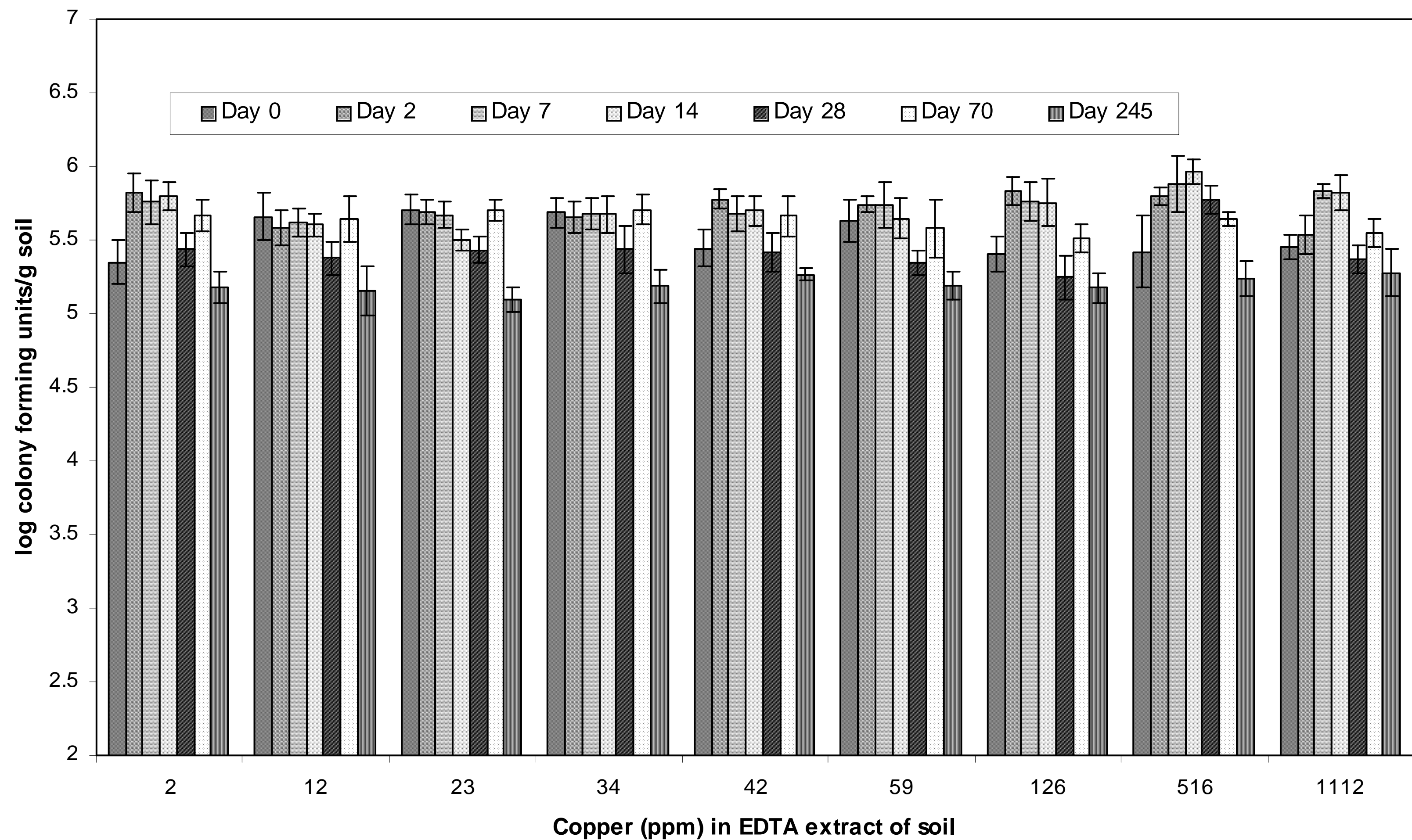

FIG 2.7. The number of filamentous fungi present in the soil microcosms treated with diffrent copper concentrations periodically enumerated on MEAs-medium.

Each bar represents the mean of three repetitions and standard deviations are indicated on top of each bar.

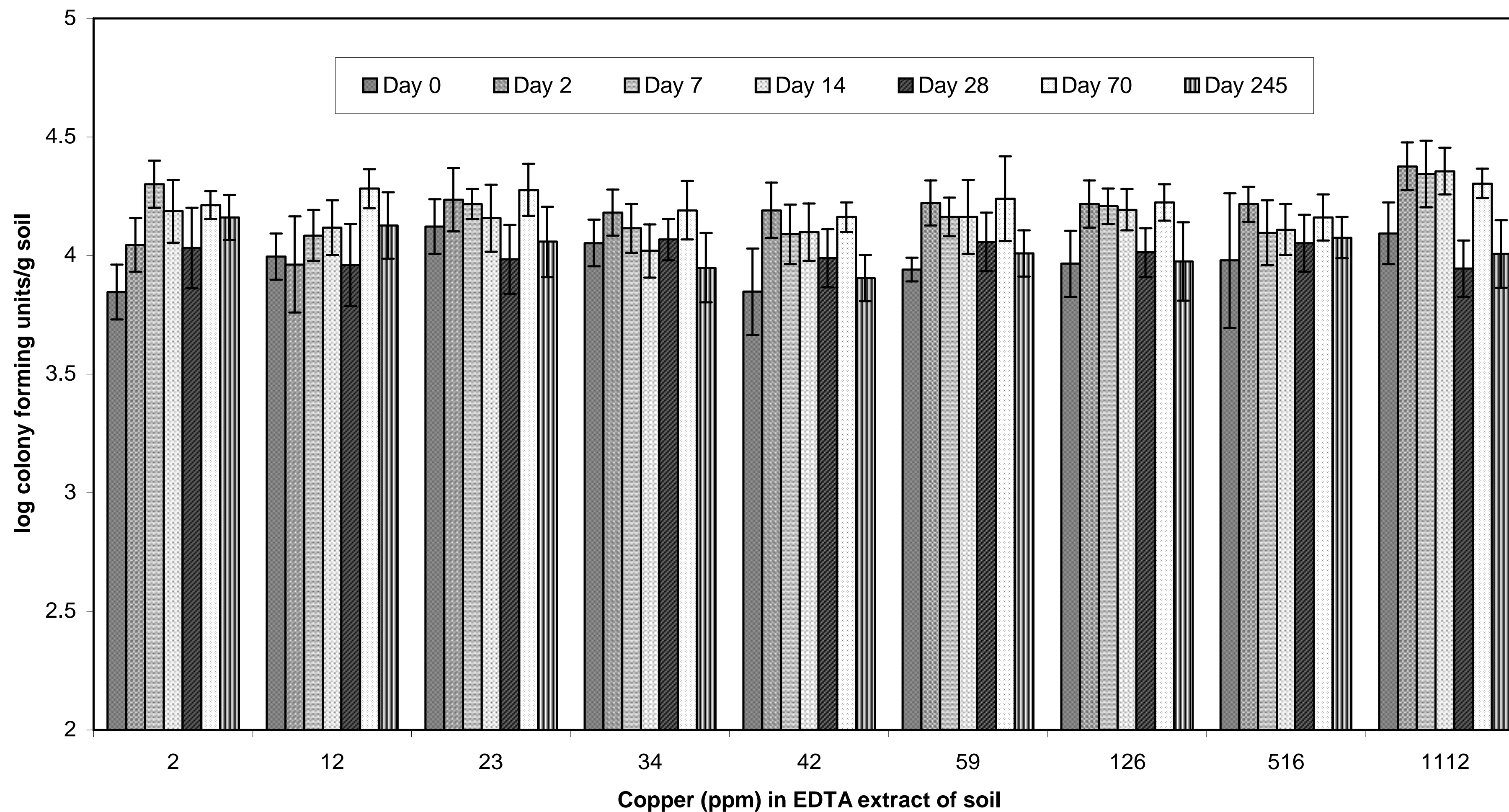

FIG 2.8. The number of hymenomycetes fungi present in the soil microcosms treated with different copper concentrations periodically enumerated on BDS-medium.

Each bar represents the mean of three repetitions and standard deviations are indicated on top of each bar.

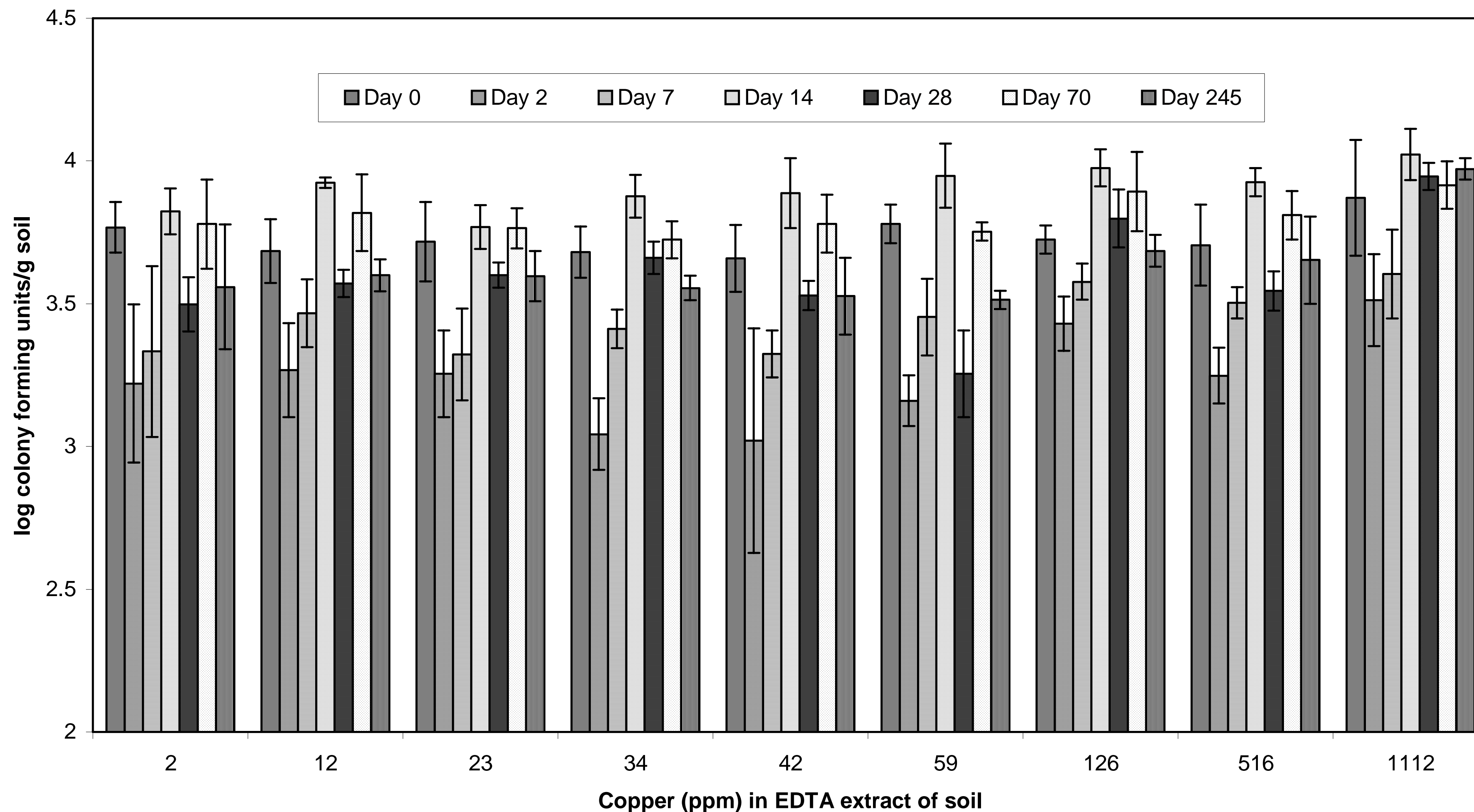

FIG 2.9. Mucoralean fungi present in the soil microcosms treated with different copper concentrations periodically enumerated on MucA-medium.

Each bar represents the mean of three repetitions and standard deviations are indicated on top of each bar.

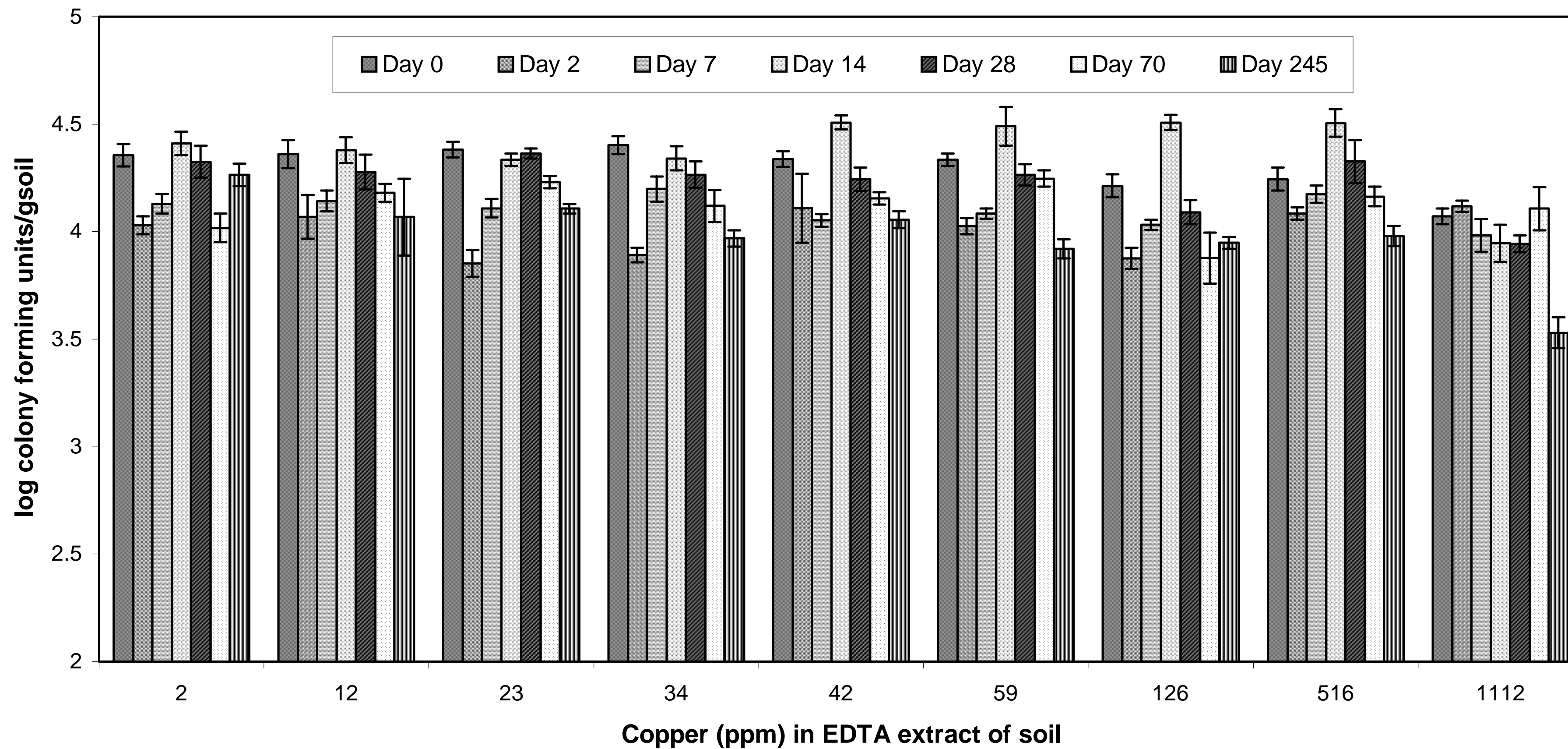

FIG 2.10. Soil yeasts present in the soil microcosms treated with different copper concentrations periodically enumerated on TMV-medium.

Each bar represents the mean of three repetitions and standard deviations are indicated on top of each bar.

To determine whether the numbers of Cu resistant fungi in the microcosms, which received additional Cu, differed from the numbers of these fungi in the control microcosms, additional plates containing 32 ppm Cu were used to enumerate fungi able to grow on each of the above mentioned media. These fungi were enumerated after 0, 14, 70 and 245 days of incubation. The percentage Cu resistant fungi in each microcosm, able to grow on each of the above mentioned media in the presence of 32 ppm Cu, was subsequently calculated as a fraction of the total number of fungi enumerated on the particular agar medium containing only spore quantities of Cu (Figs 2.11, 2.12, 2.13, and 2.14).

Generally, it was found that the percentage Cu resistant filamentous and mucoralean fungi, in microcosms that received additional Cu, were not significantly different from the percentages of these fungi in the control microcosms based on the standard deviations of the graphs (Figs 2.11 and 2.12). However, a tendency was observed among the filamentous fungi challenged with higher Cu concentrations that showed a decrease in the number of Cu resistant fungi towards the end of the incubation period. This suggested that Cu resistant fungi either produced less spores towards the end of the incubation period or fell prey to Cu resistant predators, or were out competed by other microbes. The mucoralean fungi showed an increase in Cu resistance towards the end of the incubation period in comparison with the control microcosm. The percentage Cu resistant hymenomycetous fungi generally showed a large decrease in numbers between the first two days of incubation, except at the microcosm containing 1112 ppm Cu, where no significant differences in fungal numbers were observed (Fig 2.13).

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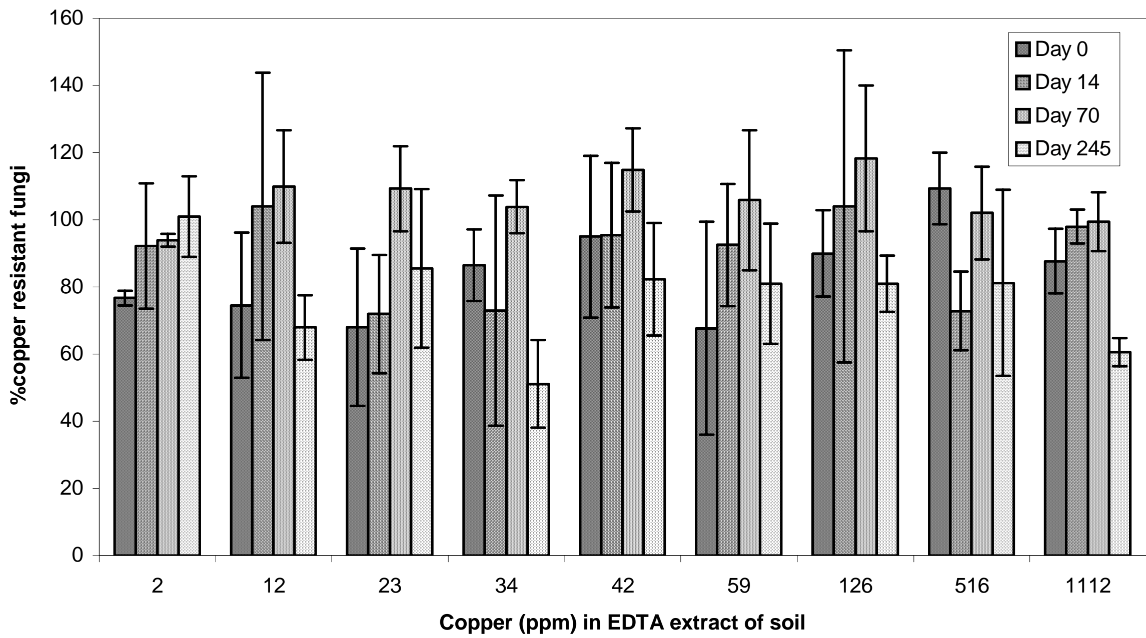


***FIG 2.11.** The percentage copper resistant filamentous fungi enumerated on the general isolation medium MEAs and MEAs containing additional copper.

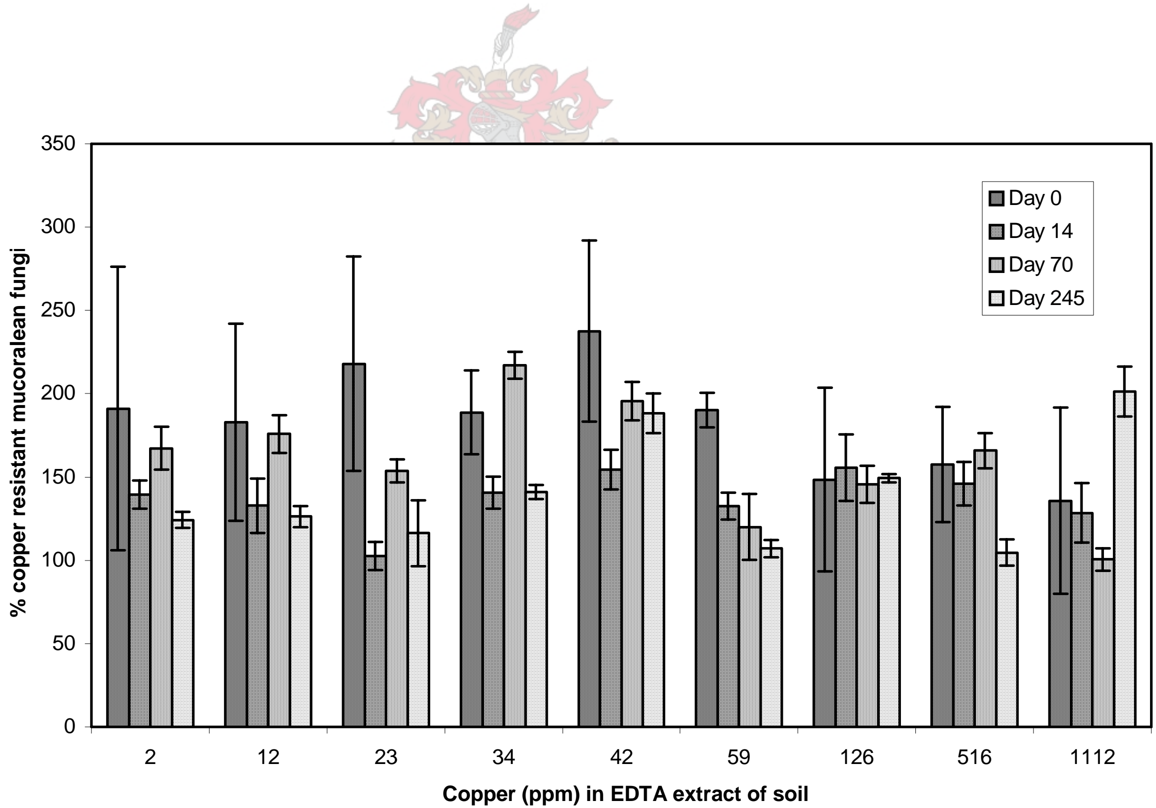


***FIG 2.12**. The percentage of copper resistant mucoralean fungi enumerated on MucA-medium and copper containing MucA.

* The standard deviation is indicated on top of each bar that in turn represents the mean of three repetitive counts.

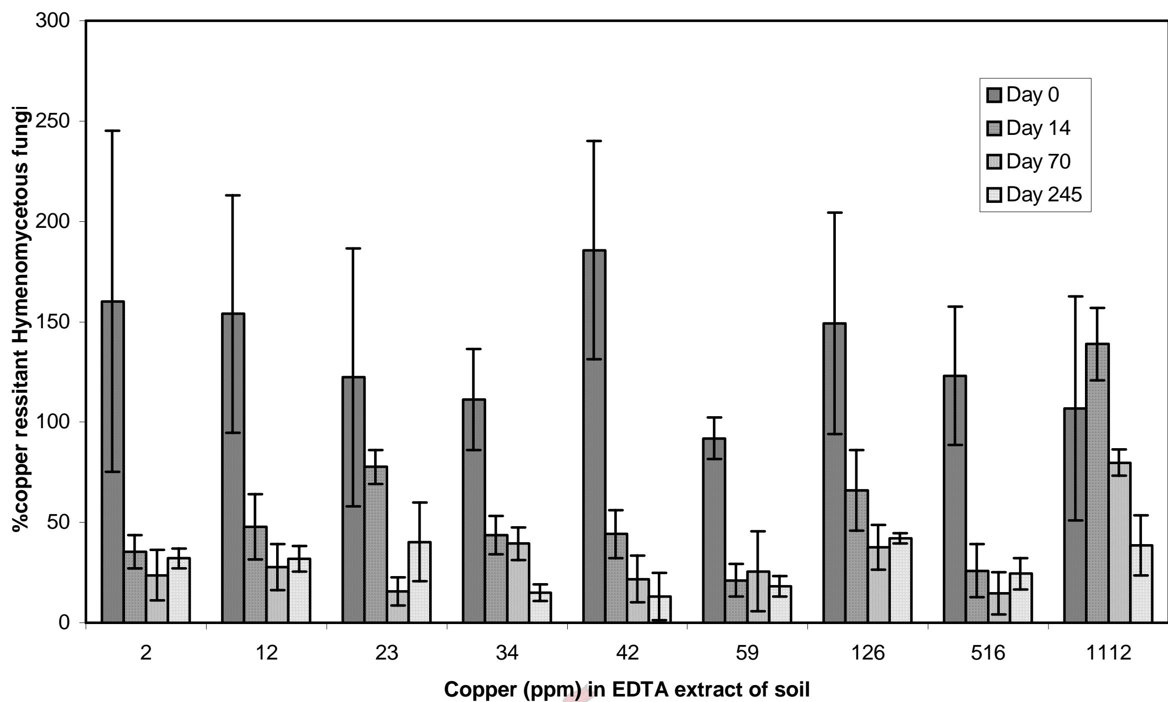
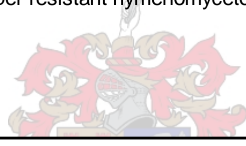


***FIG 2.13**. The percentage of copper resistant hymenomycetous yeasts enumerated on BDS -medium and copper containing BDS.



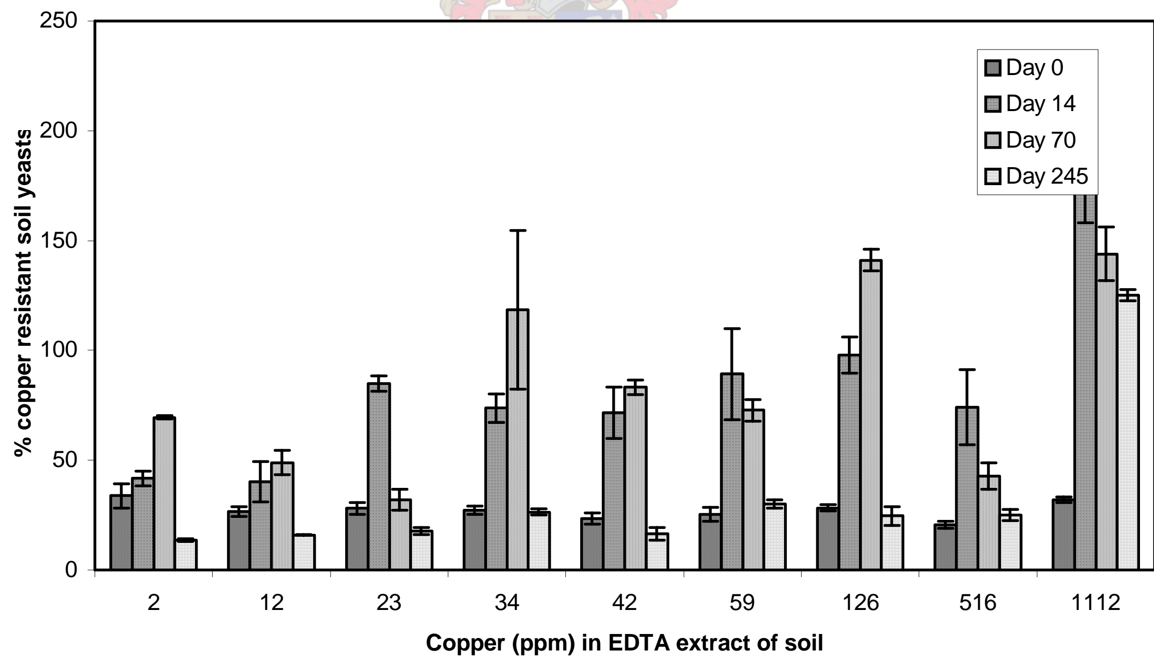


***FIG 2.14**. The percentage of copper resistant yeasts periodically enumerated on TMV-medium and copper containing TMV-medium.

* The standard deviation is indicated on top of each bar that in turn represents the mean of three repetitive counts.

The most notable changes in Cu resistance among soil fungi were observed in the yeast population (Fig 2.14). The initial percentage Cu resistant yeasts in all the soil microcosms was *circa* 30 %. During the incubation period, this figure increased and peaked, especially in the microcosms that were challenged with higher Cu concentrations. At the end of the incubation period, the percentage Cu resistant yeasts in most microcosms returned to nearly similar levels than the control. This may indicate that by this time, the competitors and/or predators of these Cu resistant yeasts have adapted to the elevated Cu levels in the soil. However, it seems that in microcosms challenged with *circa* 1000 ppm Cu, the activities of these competitors and/or predators did not recover to the same level as in the control.

2.3.5. Impact of increased soil copper concentrations on fungal biomass.

The above-mentioned results revealed the impact of soil Cu levels on selected populations of fungi able to grow on different isolation media. To obtain an indication of the impact of Cu on the total soil fungal biomass, sterol analyses of the soil microcosms were performed (Figure 2.15). The fungal sterol ergosterol (West *et al.*, 1987) was present in all the microcosms. The Student-T test (Schefler, 1979) was performed to determine the significance of the differences between the data but no significant difference (p value < 0.5) was detected between the control microcosm and the microcosms that received additional Cu.

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2.4. CONCLUSIONS

Although the Cu concentrations in the virgin soil were less than 2 ppm, filamentous fungi and yeasts, able to tolerate up to 100 ppm Cu in a growth medium occurred in the soil. Also, viable fungi were isolated from Cu contaminated soil microcosms containing as high as 1112 ppm bio-available Cu, as determined as an EDTA extract of the soil. The numbers of filamentous fungi in the soil, including the Mucorales and hymenomycetes, seemed to be less affected by the addition of copper oxychloride than the numbers of soil yeasts able to grow on TMV-agar.

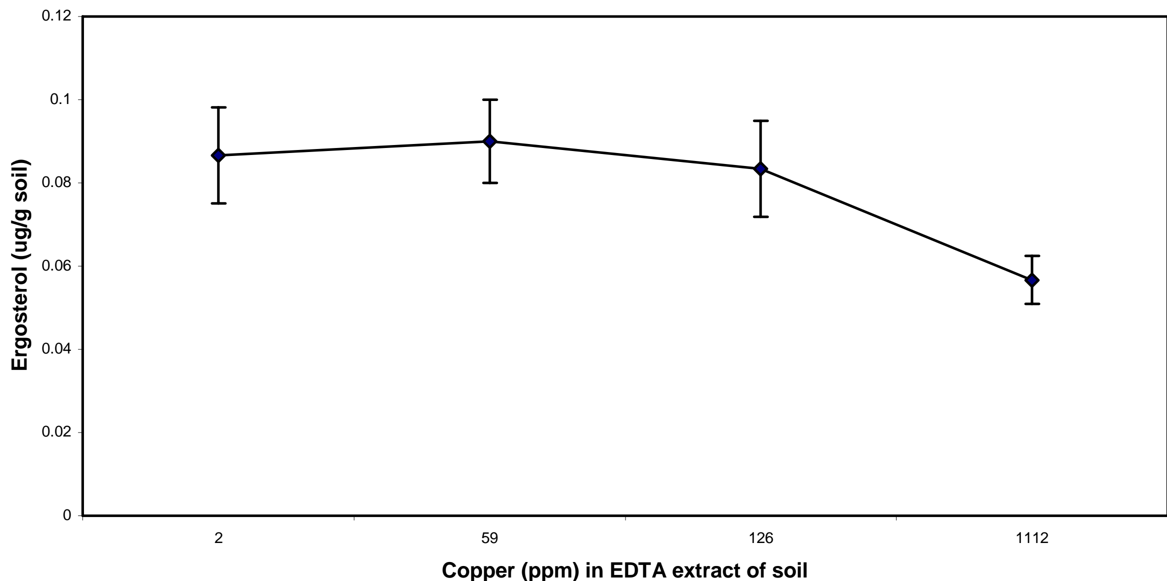

FIG 2.15. The total amount of ergosterol found per soil sample.

The numbers of these soil yeasts were initially negatively affected by the addition of copper oxychloride. Upon further incubation, the yeast numbers in most copper oxychloride containing microcosms recovered to the same level as that of the control. In addition, the percentage Cu resistant yeasts increased in these microcosms. This indicates that Cu resistance of individual strains increased in the presence of elevated Cu levels and/or that shifts in the soil yeast populations may have taken place.

The focus of the next chapter will be the response of yeasts in different soils to elevated levels of Cu in the soil. We will test the hypothesis that in addition to the inherent responses that may take place, such as decreased sensitivity to Cu by an individual strain, shifts in community composition play an important role in the maintenance of yeast activity in soil.

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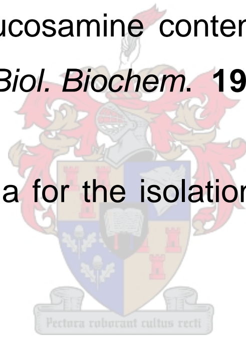

Chapter 3

Shifts in community composition provide a mechanism for maintenance of soil yeast activity in the presence of elevated copper levels

This chapter has been published in Canadian Journal of Microbiology, 49: 425 – 432 (2003).

3.1. INTRODUCTION

Numerous workers studied the impact of pollutants and agricultural chemicals, containing heavy metals such as copper (Cu), on soil microbes. Sandaa *et al.*, (1999) found that bacterial diversity, as well as bacterial community structure in soil is sensitive to elevated levels of heavy metals while filamentous fungi were found to be less susceptible than prokaryotes (Doelman, 1985; Hiroki, 1992). Many in vitro studies were conducted on the molecular and cellular responses to heavy metals in fungi including yeasts (Fogel *et al.*, 1983; Oh *et al.*, 1999). In addition, the impact of heavy metals on filamentous fungal populations in soil was studied and it was found that increased concentrations of these metals might affect both the qualitative and quantitative composition of the populations (Wainwright and Gadd, 1997). Some studies also indicated that the survival of filamentous fungi in the presence of elevated soil Cu concentrations is dependent on the inherent properties of the organisms rather than on adaptive changes occurring over time. However, relatively little is known about the impact of Cu on the yeast populations in soil.

We are interested in elucidating strategies by which soil microbial communities maintain their overall activity during fluctuations in their chemical/physical environment. In this study the focus was on the response of yeasts in different soils to elevated levels of Cu in the soil. Such information is relevant especially in wine-producing regions where Cu-based fungicides are widely used. We tested the hypothesis that in addition to the inherent responses that may take place, such as decreased sensitivity to antimicrobial molecules by an individual strain, shifts in community composition play an important role in the maintenance of yeast activity in soil. Subsequently, TMV-agar (Chapter 2) was used to monitor a diverse group of unrelated yeasts in soil microcosms prepared from four soil samples, which were experimentally treated with the fungicide, copper oxychloride.

3.2. MATERIALS AND METHODS

3.2.1. Selective medium used for yeast enumeration

TMV-agar (Chapter 2) was used to enumerate soil yeasts. The composition of this medium is depicted in Table 3.1.

3.2.2. Determining the impact of copper oxychloride on soil yeasts.

Soil was taken from four different sampling sites in the Western Cape, South Africa. After the organic matter at the surface was removed, the top 30 cm of soil was collected. Approximately 600 kg of each soil type was allowed to dry for 2 weeks at 30 °C, after which it was sieved (pore size 2 mm) and the whole batch thoroughly mixed to ensure homogeneous subsampling. The clay content, organic carbon and pH of the sieved soil originating from each sampling site are listed in Table 3.2.

A series of soil microcosms was prepared from each soil sample by adding a different concentration of copper oxychloride ($CuCl_2 3Cu(OH)_2$) to sub-samples of the soil. Each of these sub-samples was further divided into triplicate microcosms in plastic soil bags, each containing ~ 1.5 kg soil. The total concentration of administered and the final concentration of bio-available Cu in each triplicate, as determined in a di-ammonium EDTA extract of the soil (Beyers and Coetzer, 1971) are depicted in Table 3.3.

Distilled water was added to each microcosm one day after addition of Cu to result in soil moisture content of 15% (v/w). Thereafter 200 ml water per microcosm was added every two weeks. The microcosms were incubated at 22°C. To enumerate yeasts, soil dilution plates using TMV-agar were periodically prepared. Yeast colonies, ranging from one millimetre and more in diameter, were counted after one week of incubation at 22°C. Cu resistant yeasts were enumerated on TMV-agar containing 0.5 mM $CuSO_4$ (TMVc-agar). This concentration of Cu (32 ppm) is known to select for Cu resistant yeasts (Thiele, 1988).

Yeasts from Soil Sample IV were enumerated on TMV-agar and TMVc-agar after an incubation period of 84 days. To evaluate shifts in species composition as a result of the additional Cu these yeasts were randomly isolated from plates prepared from microcosms that received zero (control) and ~ 1000 ppm Cu. These random samples of yeast isolates were obtained using a modification of the Harrison's disc method as described by Harrigan and McCance (1967). Successive inoculation and incubation on MEA at 22°C were used to purify the isolates.

Subsequently, the isolates were identified by analysing the nucleotide sequences of the variable D1/D2 domains of the large subunit (26S) of ribosomal DNA. Yeast isolates were grown for 24h in 20 ml of yeast-peptone-dextrose (YPD) broth (2% glucose, 2% peptone, 1% yeast-extract). Genomic DNA was extracted according to the method of Hoffman and Winston (1987). The D1/D2 600-650bp region of the large subunit of ribosomal DNA (rDNA) was subsequently amplified using the polymerase chain reaction (PCR). DNA was amplified using the forward primer F63 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and the reverse primer LR3 (5'-GGT CCG TGT TTC AAG ACG G-3') in a Perkin-Elmer thermal cycler (Fell *et al*., 2000). The PCR products were purified with Nucleospin® (Separations) chromatography columns. Sequences representing the D1/D2 region of the rDNA from the strains were obtained using an ABI PRISM model 3100 genetic sequencer. The forward and reverse sequences were aligned with DNAMAN for WINDOWS Version 4.13 (Lynnon Biosoft). The yeast isolates were identified by comparing the sequencing results with known sequences using the BLAST program (www.ncbi.nlm.nih.gov/blast).

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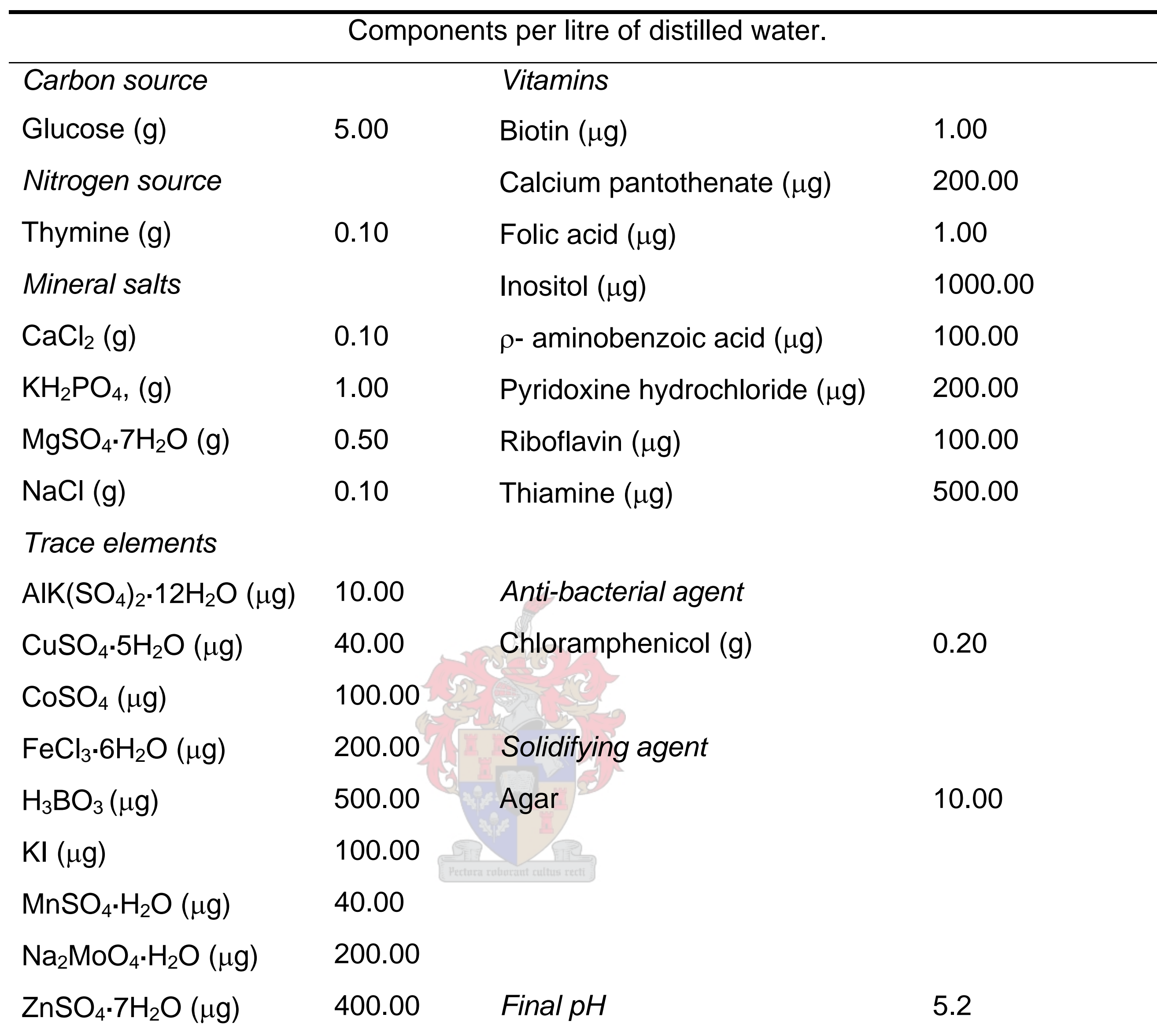
Table 3.1. The composition of thymine-mineral-vitamin-agar (TMV-agar) used to isolate lipomycetaceous yeasts from soil (Mothibeli, 1996) and subsequently used as enumeration medium in this study (Chapter 2).



Components per litre of distilled water.			
Carbon source		*Vitamins*	
Glucose (g)	5.00	Biotin (μg)	1.00
Nitrogen source		Calcium pantothenate (μg)	200.00
Thymine (g)	0.10	Folic acid (μg)	1.00
Mineral salts		Inositol (μg)	1000.00
$CaCl_2$ (g)	0.10	ρ- aminobenzoic acid (μg)	100.00
KH_2PO_4, (g)	1.00	Pyridoxine hydrochloride (μg)	200.00
$MgSO_4 \cdot 7H_2O$ (g)	0.50	Riboflavin (μg)	100.00
NaCl (g)	0.10	Thiamine (μg)	500.00
Trace elements			
$AlK(SO_4)_2 \cdot 12H_2O$ (μg)	10.00	*Anti-bacterial agent*	
$CuSO_4 \cdot 5H_2O$ (μg)	40.00	Chloramphenicol (g)	0.20
$CoSO_4$ (μg)	100.00		
$FeCl_3 \cdot 6H_2O$ (μg)	200.00	*Solidifying agent*	
H_3BO_3 (μg)	500.00	Agar	10.00
KI (μg)	100.00		
$MnSO_4 \cdot H_2O$ (μg)	40.00		
$Na_2MoO_4 \cdot H_2O$ (μg)	200.00		
$ZnSO_4 \cdot 7H_2O$ (μg)	400.00	*Final pH*	5.2

Table 3.2. [a]Clay content and pH of the soil samples used in the experimentation

Characteristics	*Soil Sample I*	*Soil Sample II*	*Soil Sample III*	*Soil Sample IV*
[b]Clay (%)	3.80	8.90	5.10	21.80
[c]Organic Carbon (%)	0.75	1.61	1.71	1.50
[d]pH (KCl).	5.10	4.30	4.70	5.90

[a] For a detailed summery of the chemical and physical characteristics of these soil samples, see Du Plessis (2002).

[b] Determined using the hydrometer method (Van der Watt 1966).

[c] Determined by using the Walkey-Black method (Nelson and Sommers 1982).

[d]Determined according to the method of McClean (1982).

Note: *Soil Sample I,* potting soil prepared at an experimental farm near Stellenbosch, South Africa; *Soil Sample II,* vineyard soil obtained from a commercial wine farm near Stellenbosch, South Africa; *Soil Sample III,* pristine soil originating from the indigenous fynbos vegetation near Stellenbosch, South Africa*; Soil Sample IV,* vineyard soil obtained from a commercial wine farm near Somerset West, South Africa.

3.2.3. Determining the level of Cu tolerance of yeast isolates

A 6.7 g/l Yeast Nitrogen Base (YNB, Difco) plus 20 g/l glucose medium was used to prepare 14 h-old cultures of each isolate. Subsequently, 40 μl of each culture was used to inoculate a series of 14 test tubes each containing 5 ml of the same synthetic liquid medium as describe above, but with different concentrations of $CuSO_4{\cdot}5H_2O$. The final Cu concentration in each series was 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 and 500 ppm. The inoculated tubes were incubated at 22°C on a rollordrum rotating at 100 rpm. After nine days the presence of growth in each tube was determined using the method of Van der Walt and Yarrow (1984). All these experiments were conducted in triplicate.

Table 3.3. Concentration of Cu (ppm) in a di-ammonium EDTA extract of the soil in each microcosm prepared from the different soil samples.

[a] Cu administrated	Soil Sample I [b]Cu in EDTA	Soil Sample II Cu in EDTA	Soil Sample III Cu in EDTA	Soil Sample IV Cu in EDTA
0	0.83 (±0.03)	3.14 (±0.31)	0.76 (±0.11)	1.05 (±0.06)
10	11.65 (±2.03)	10.06 (±1.11)	6.43 (±0.97)	11.84 (±0.9)
30	29.47 (±1.96)	19.06 (±1.04)	15.34 (±1.01)	25.31 (±0.65)
50	48.95 (±6.96)	31.62 (±1.33)	25.00 (±1.03)	43.58 (±2.85)
100	101.8 (±9.40)	60.14 (±4.74)	56.75 (±1.02)	89.57 (±1.01)
500	422.6(±9.98)	327.28 (±13.72)	313.56 (±5.97)	477.5 (±16.87)
1000	1008.15 (±141.81)	608.17 (±26.02)	652.4925 (±25.74)	973.3 (±14.76)

[a] Cu concentration (ppm) administered to the soil as copper oxychloride.

[b] Cu concentration (ppm) determined in a di-ammonium EDTA extract according to the methods of Beyers and Coetzer (1971).

Note: The numbers represent the means and standard deviation (in brackets) of three repetitions; ND, not determined.

3.2.4. Statistical analyses

The data from the experimental design (four random soil samples, each sub-sampled and treated in triplicate with increasing levels of Cu) were analysed using STATISTICA version 6. The analyses were performed on observations made in triplicate for the soil samples, at three time intervals (after 1 and 14 days, and at the end of the incubation period), as well as for seven different concentrations of administered Cu (Table 3.3). Where application of ANOVA revealed that the factors had a significant effect on yeast numbers or the percentage Cu resistant yeasts, the differences between treatments were further separated at $p \leq 0.05$, using a post hoc least significant difference (LSD) multiple comparison test, Tukey's HSD tests, as well as Bonferroni (Dunn) t tests.

3.3. RESULTS AND DISCUSSION

3.3.1. Determining the impact of copper oxychloride on soil yeasts

The soil yeast counts in microcosms prepared from the four soil samples are depicted in Fig 3.1 *a* to *d*. This data revealed that the soil yeast numbers did not change significantly ($p \leq 0.05$) in response to the Cu administered to the soil. Statistical analyses further demonstrated that, when analyzed together, there was no significant variation in yeast numbers in the different soils over time. In contrast, there were differences in yeast numbers in the different soils – while there was no significant difference between the number of yeasts in soils II and III, they differed from soils I and IV ($p \leq 0.05$). These differences may be ascribed to differences in the chemical and physical characteristics of the soils.

Although the yeast numbers did not show significant variation over the duration of experimentation, a few trends were observed. For instance, at the beginning of the incubation period, there was a decrease in yeast numbers in all microcosms prepared from Soil Sample I and treated with Cu (Fig. 3.1*a*). This phenomenon did not occur in the other series of microcosms prepared from Soil Samples II, III and IV (Fig 1. *b, c* and *d*). This may be ascribed to the higher clay and organic matter content of the latter samples (Table 3.2). It is known that these soil components adsorb divalent cations (McBride 1994), which may have resulted that the toxic effects of Cu on the soil microbes were attenuated by the presence of clay and / or organic matter in the soil. There was a decrease in yeast cell numbers in microcosms prepared from Soil Samples II and III that received higher Cu concentrations (Fig. 3.1 *b* and *c*) after 14 days. This delayed negative impact of Cu on the yeast numbers was evident in microcosms containing more than 15 ppm Cu in the EDTA extract of the soil. No such negative impact was observed in microcosms prepared from Soil Sample IV (Fig. 3.1 *d*). After 14 days of incubation, there was an increase in yeast numbers in the copper oxychloride containing microcosms prepared from Soil Sample I (Fig. 3.1*a*).

Towards the end of the incubation period, the yeast numbers in all the microcosms with more than 314 ppm Cu in the EDTA extract of the soil had recovered from the initial apparent negative impact of the Cu (Fig. 3.1 *a* - *d*). The yeast numbers in Soil Samples II, III and IV were even higher than in the control (Fig. 3.1 *b, c*, and *d*). This may be as a result of a negative impact of these higher Cu concentrations on the competitors and/or protozoan predators of the soil yeasts. It is known that, because of their delicate external membranes, protozoa are highly sensitive for elevated concentrations of heavy metals (Foissner, 1994).

The natural Cu concentrations in all the soil samples were less than 3.2 ppm (Table 3.3). At the start of the incubation period, a substantial proportion of the yeast population in the control microcosms of all the soil samples was able to grow on TMVc-agar containing 32 ppm Cu. The percentages of these Cu resistant yeasts in the control microcosms of the different soil samples were respectively 23% (Soil Sample I); 58% (Soil Sample II); 54% (Soil Sample III) and 37% (Soil Sample IV). However, statistical analyses revealed that the soil type had no significant effect on the relative numbers of Cu resistant yeasts in the microcosms following Cu administration. In contrast, the amount of Cu administered had a significant effect ($p \leq 0.05$) on the percentages of Cu resistant yeasts in the soil, where increases in Cu concentration resulted in increases in the percentage Cu resistant yeasts. The time to respond to Cu was not constant in all soil samples. For instance, after 14 days of incubation, the proportion of Cu resistant yeasts increased in all the Cu-challenged microcosms prepared from Soil Sample I (Fig 3.2 *a*), while those prepared from Soil Samples II, III and IV, only showed increases in the percentage Cu resistant yeasts after incubation longer than 14 days, especially in microcosms containing more than 314 ppm Cu in the EDTA extract of the soil (Fig 3.2 *b, c*, and *d*). Interestingly, such increases were not observed in the total soil yeast numbers (Fig 3.1 *a* – *d*), giving support to the hypothesis that community shifts may play an important role in maintaining overall soil yeast activity.

To further evaluate this, yeast colonies originating from Soil Sample IV were randomly isolated and identified from microcosms challenged with zero (control) and ~1000 ppm additional Cu, after incubation for 84 days. The isolates were

representatives of the Ascomycetes, Hymenomycetes and Urediniomycetes, confirming that a diversity of yeast taxa is able to grow on TMV-agar (Tables 3.4 and 3.5). It must however be noted that the isolates represent genera not known for the utilization of heterocyclic nitrogen containing compounds (Phaff and Starmer, 1987; Barnett *et al*., 2000). The yeast growth observed on the TMV-agar was most probably a result of the ability of the yeasts to grow under oligonitrotrophic conditions, since representatives of *Cryptococcus* and *Rhodotorula* were found by other workers to grow oligotrophically (Kimura *et al*., 1998).

When the yeasts that were isolated in this study from microcosms challenged with zero and ~1000 ppm Cu were exposed to different Cu concentrations in YNB liquid medium, it was found that intra-specific differences as well as differences within the above mentioned classes, did exist regarding Cu tolerance (Tables 3.4 and 3.5). The level of Cu tolerance of a particular isolate did not always correlate with the origin of the isolate (treated or not treated with Cu). Also, in a number of cases, the level of Cu tolerance far exceeded the Cu concentrations the yeasts were challenged with on the isolation plates and in the microcosms. For example, yeasts were isolated from the control microcosm able to grow in the liquid medium containing at least 500 ppm Cu. When ~ 1000 ppm Cu was added to the soil, it was found that regardless of the level of Cu tolerance of the yeasts, there were relatively more urediniomycetous yeasts compared to hymenomycetous species (Tables 3.4 and 3.5).

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The results discussed above are in accordance with the work of Arnebrant *et al.* (1987), who found that intra-specific adaptation of filamentous fungi to high soil Cu levels plays a minor role in the occurrence of Cu tolerant fungi in soil contaminated with this heavy metal. The high amount of Cu tolerant filamentous fungi in contaminated soil rather reflected shifts in species composition, similar to what was found in our study on yeasts.

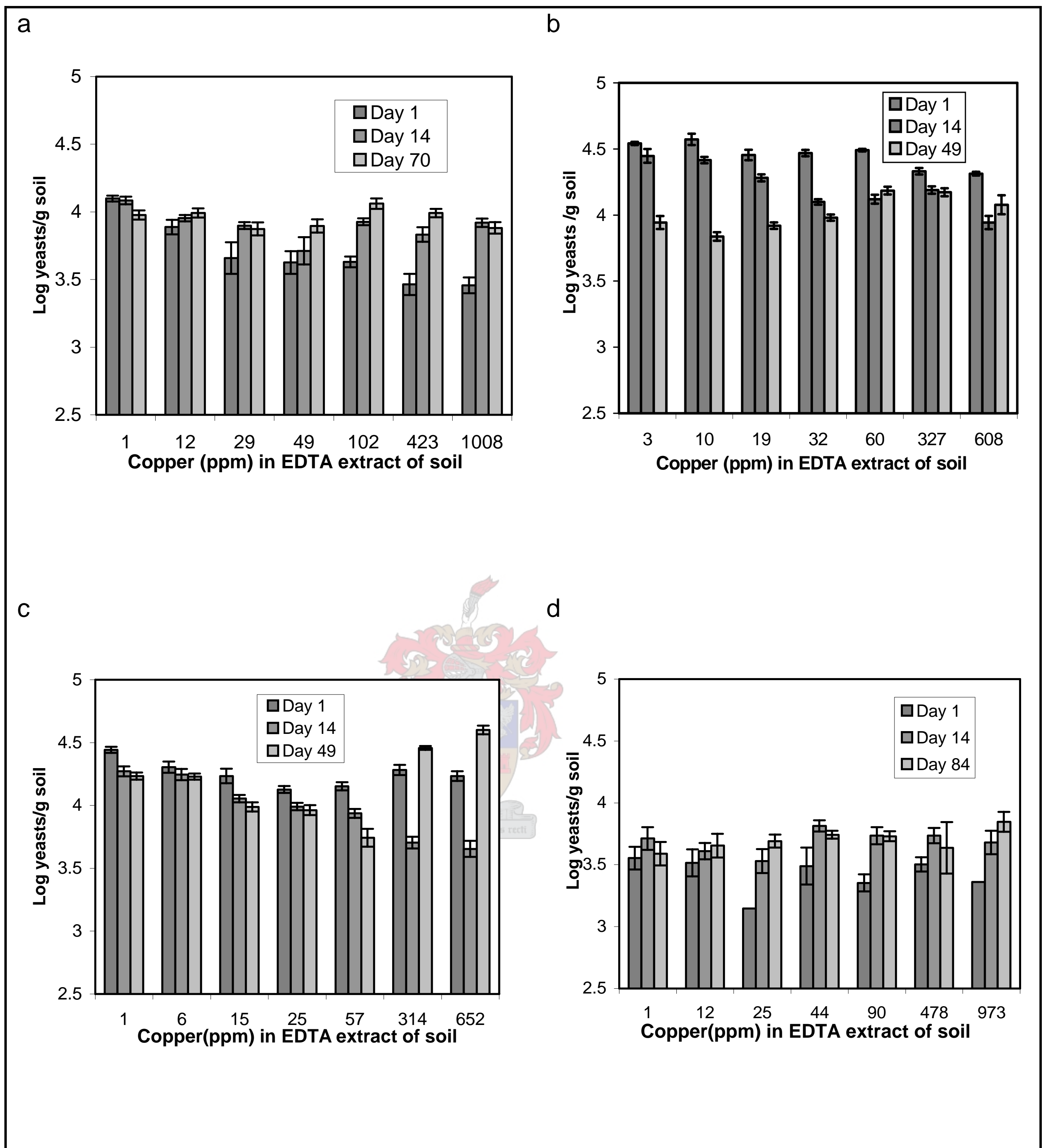

FIG 3.1. Soil yeast numbers in microcosms as enumerated on TMV-agar. Graphs represent the data obtained for Soil Sample I (*a*), Soil Sample II (*b*), Soil Sample III (*c*) and Soil Sample IV (*d*). Bars represent means and standard deviation for triplicate counts.

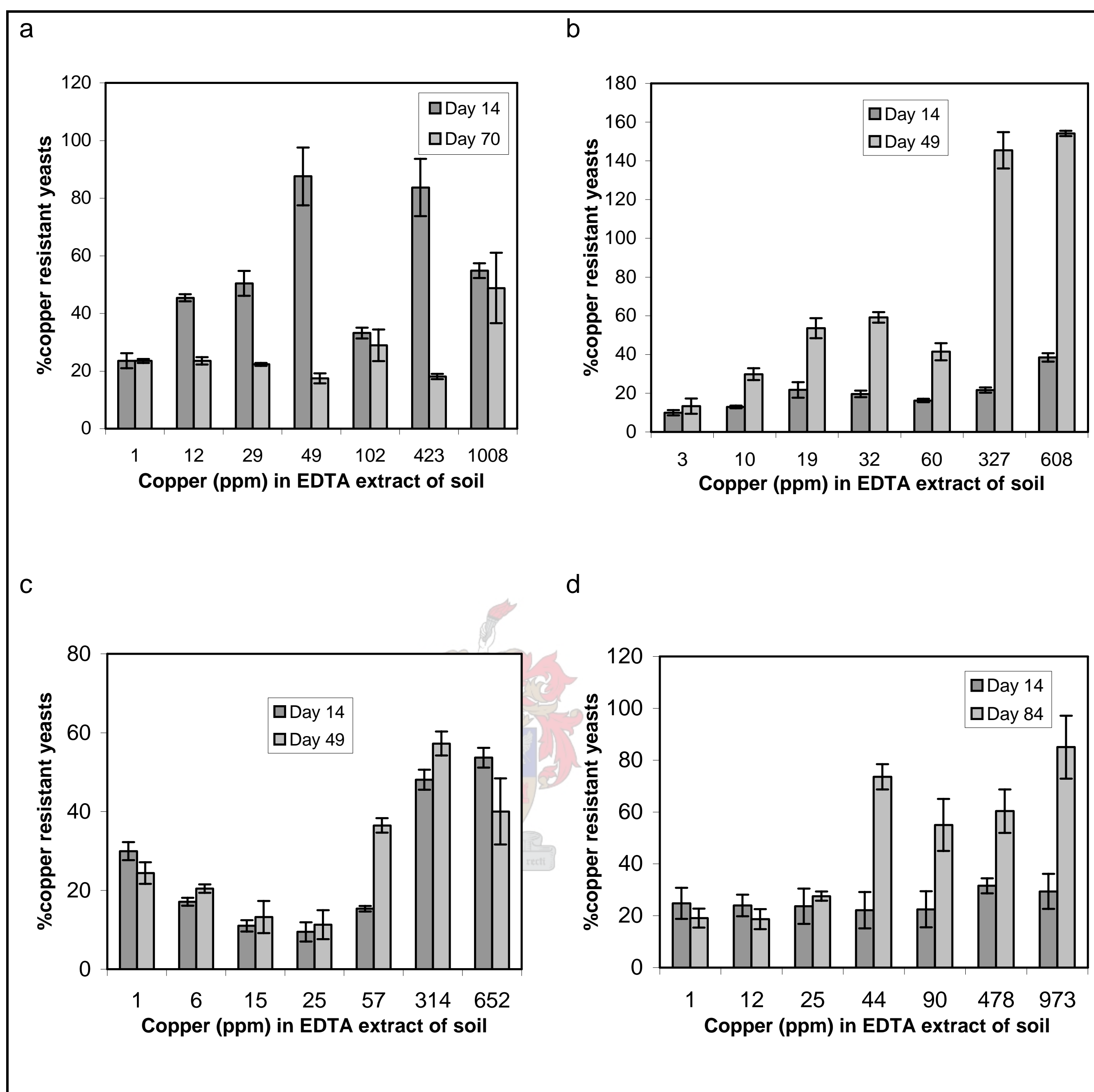

FIG 3.2. Relative abundance of Cu resistant yeasts occurring in the microcosms and able to grow on TMVc-agar, calculated as a percentage of the total number of isolates enumerated on TMV-agar. Graphs represent data obtained for Soil Sample I (*a*), Soil Sample II (*b*), Soil Sample III (*c*) and Soil Sample IV (*d*). Bars represent means and standard deviation for triplicate counts.

Table 3.4. Identity and Cu tolerance of yeast species enumerated on TMV-agar after 84 days of incubating the control and the microcosms containing *ca*. 1000 ppm Cu.

Species	Control microcosm		Microcosm challenged with *ca*. 1000 ppm Cu.	
	Relative abundance of different species as percentage of the total number of isolates.	[a]Cu tolerance	Relative abundance of different species as percentage of the total number of isolates	[a]Cu tolerance
Hymenomycetes				
Cryptococcus arrabidensis	7.9	⊥	-	-
Cryptococcus phenolicus	46.1	⊥/++	28.5	⊥/++
[b]*Cryptococcus* species 1	15.4	++++	10.7	++++
[c]*Cryptococcus* species 2	15.4	++++	-	-
Tremella globispora	7.9	++++	-	-
Urediniomycetes				
Rhodotorula graminis	7.9	++++	7.1	++++
Rhodotorula laryngis	-	-	3.6	+

Table 3.4. (Continued)

Rhodotorula minuta	-	-	28.5	+
Rhodotorula nothofagi	-	-	14.3	+
Rhodotorula slooffiae	-	-	7.1	+
	100		100	
Log of total number of yeasts / g soil	3.60		3.85	

[a]Tolerance measured as growth in tubes containing liquid media with different concentrations of copper sulphate: ⊥= all isolates showed growth in the presence of 50 ppm Cu. += all isolates showed growth in the presence of up to 100 ppm Cu; ⊥/++= isolates showed growth in the presence of up to 50 ppm, 100 ppm Cu or 200 ppm Cu; ++++= all isolates showed growth in the presence of up to 500 ppm Cu.

[b]Isolate proved to be identical to *Cryptococcus* KCTC 17078, GenBank accession no. AF459694.

[c]Isolate proved to be identical to *Cryptococcus* CBS 8372, GenBank accession no. AF444410.

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Table 3.5. Identity and Cu tolerance of yeast species enumerated on TMVc-agar containing 32 ppm Cu after 84 days of incubating the control and the microcosms containing *ca*. 1000 ppm Cu.

Species	Control microcosm		Microcosm challenged with ca. 1000 ppm Cu.	
	Relative abundance of different species as percentage of the total number of isolates	[a]Cu tolerance	Relative abundance of different species as percentage of the total number of isolates.	[a]Cu tolerance
Hymenomycetes				
Cryptococcus albidus	6.3	++++	-	-
Cryptococcus bhutanensis	12.6	+	-	-
Cryptococcus podzolicus	-	-	14.3	++++
[b]*Cryptococcus* species 2	37.9	++++	-	-
Urediniomycetes				
Leucosporidium scottii	-	-	14.3	+
Rhodotorula graminis	-	-	7.1	++++

Table 3.5. (Continued)

Rhodotorula minuta	25.3	+/++	50.0	+/+++
Rhodotorula nothofagi	6.3	⊥	7.1	+
Rhodotorula slooffiae	-	-	7.1	++
Ascomycetes				
Candida guilliermondii	12.6	++	-	-
	100		100	
Log of total number of yeasts / g soil	2.87		3.78	

[a]Tolerance measured as growth in tubes containing liquid media with different concentrations of copper sulphate: ⊥ = all isolates showed growth in the presence of 50 ppm Cu. + = all isolates showed growth in the presence of up to 100 ppm Cu; +/++ = isolates showed growth in the presence of up to 100 ppm or 200 ppm Cu; +/+++ = isolates showed growth in the presence of up to 100 ppm, 150 ppm or 250 ppm Cu; ++++ = all isolates showed growth in the presence of up to 500 ppm Cu.

[b]Isolate proved to be identical to *Cryptococcus* CBS 8372, GenBank accession no. AF444410.

3.4. CONCLUSIONS

Using TMV-agar as enumeration medium, it was found that the addition of copper oxychloride to soil had no significant impact on total yeast numbers in the soil. Yeasts that were able to grow in the presence of Cu at concentrations much higher than what occurred in their natural habitat, were present in all four soil types used in these experiments. Although the total numbers of yeasts in the different soils did not correlate with the concentration of administered Cu in the soil, the relative abundance of Cu resistant yeasts did. This may be indicative that the composition or survival of soil yeast communities in the presence of elevated Cu concentrations depends not solely on the development of tolerance by all community members following exposure to Cu. In fact, the apparent abundance of yeast strains with high Cu tolerance in untreated soils, and the demonstrated ease at which shifts in the yeast community occurred in this study, appear to be underlying mechanisms for survival when exposed to Cu.

The results suggest that, to the expense of hymenomycetous yeasts, members of the urediniomycetes were favoured by the presence of elevated levels of Cu in the soil, even though the urediniomycetes species were not necessarily more tolerant to Cu. This, and our observations that the administered Cu had no significant effect on total yeast numbers, supports the contention that community activity is maintained through shifts in composition. Whether this is a common phenomenon remains to be evaluated in other soil types.

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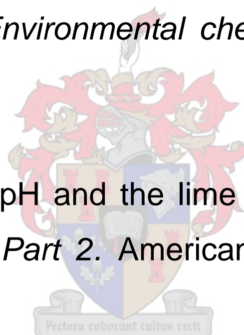

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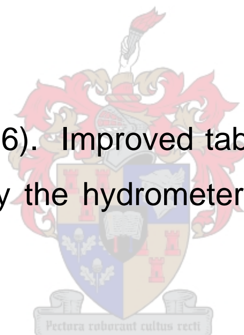

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