Application of plant growth promoting substances and arbuscular mycorrhizal fungi for phytostabilisation of mine tailings

by

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Marthinus Jacob Rossouw Date: March 2016

Abstract:

This study focused on investigating methods of phytostabilisation of mine tailings operated by Palabora Copper in South Africa. Capping material and mine tailing at various sites of the mine were collected and used in pot trials to investigate the effect of a number of plant growth promoting substances (PGPS) on several of grass species currently used in effort to stabilise the areas in question. Lumichrome, strigolactones (GR24), flavonoids (CropbioLife™), smoke-water (karrikins) and arbuscular mycorrhizal fungi (Mycoroot™) were used as PGPS to investigate growth-promoting effects on i) *Anthephora pubescens*, *Cenchrus ciliaris*, *Chloris gayana*, *Cynodon dactylon*, and *Panicum maximum* which are species currently used by Palabora Copper for rehabilitation of mine tailings, and ii) Additional grass species theoretically suited to surviving the environment. Treatments were applied on 2-week old transplanted grass seedlings in pot trials containing mine capping material as the substrate, to infer treatment responses. Trypan-Blue staining procedures were used to ascertain which grass species formed symbiotic relationship with arbuscular mycorrhizal fungi (AMF), which would potentially aid in their survival in deleterious areas. Germination rates were measured to determine the fastest germinating species of the selected grasses with *Eragrostis teff* and *Melinis repens* germinating the quickest in the mine capping material.

Capping material and mine tailing samples were collected at sites under revegetation by Palabora Copper. This included samples of the rhizosphere of locally abundant plants at two sites: a recently (two years) capped mine tailing, and a rock dump site (capped 10-12 years previously). Five rhizosphere samples were collected from individuals of *Cenchrus ciliaris*, *Enneapogon cenchroides*, and *Tephorisia polystachya* (a locally abundant forb species) at site 1 and *Cenchrus ciliaris*, *Stipagrostis hirtigluma*, *Tephrosia polystachya*, and *Pennisetum setaceum* at site 2. At both sites the soil of open areas devoid of plants was also sampled. Metagenomic DNA was extracted from the collected samples, often following enrichment techniques. Dilution series spread plates to determine culturable bacteria present in the tailing samples were also utilised. Polymerase Chain Reactions were implemented to produce amplicons of conserved regions within AMF and bacteria present in the mine tailing site. The predominant genera of bacteria detected in the collected tailing samples belonged to *Bacillus*. However, due to the use of enrichment techniques it was not possible to comment on the relative abundance of different bacteria in the environment where the samples were collected.

Due to the small-scale *ex* situ nature of the experiments the results gained from the PGPS treatment trials and microbial DNA isolation are not necessarily representative of the ecological environment present *in* situ. However, PGPS treatment of the selected grasses did not elicit any clear beneficial responses in the measured growth parameters, making application thereof of limited benefit for phytostabilisation purposes. Trypan staining revealed most of the grass species are capable of forming symbiotic relationships with mycorrhizal fungi, with trials indicating that AMF might benefit plants present in the mine tailings.

Samevatting:

Hierdie studie het gefokus op die ondersoek metodes van phytostabilisasie van mynuitskot wat bedryf word deur Palabora Copper in Suid-Afrika. Bedekkingsmateriaal en mynuitskot op verskillende terreine van die myn is versamel en gebruik in pot proewe om die rol van verbindings wat plantgroei bevorder (PGBV) op sewe gras spesies wat tans gebruik word in die stabilisering van die spesifieke gebiede te ondersoek. Lumichrome, strigolaktone (GR24), flavonoïede (CropbioLife™), rook-water (karrikins) en arbuskulêre mikorisaie swamme (Mycoroot™) is die PGBV wat gebruik was plantgroei bevorder te ondersoek in i) *Anthephora pubescens, Cenchrus ciliaris, Chloris gayana, Cynodon dactylon,* en *Panicum maximum*, wat tans gebruik word deur Palabora Copper rehabilitasie pogings, en ii) Addisionele grasspesies wat teoreties bepaal is as mees geskik om te oorleef in die omgewing was gebruik in die pot proewe. Behandelings (PGBV) is toegepas op 2 week oue oorgeplante gras saailinge in pot proewe met myn bedekkingsmateriaal, om die reaksie op die behandelinge af te lei. Trypan-Blue bevlekkking prosedures was geïmplementeer om vas te stel watter van die grasspesies simbiotiese verhoudings met arbuskulêre mikorisaie swamme (AMS) vorm en potensieel kan help in hul oorlewing in skadelike gebiede. Groeikoerse vir die grasse wat gebruik is gemeet om te bepaal watter van die gekose grasspesies groei die vinnigste. Beide *Eragrostis teff* en *Melinis repens* het die vinnigste ontkiem in die myn bedekkingsmateriaal.

Bedekkingsmateriaal en mynuitskot monsters is versamel by terreine wat gebruik word deur Palabora Copper. Dit sluit monsters in van die risosfeer van geselekteerde plante op twee plekke: 'n onlangs (twee jaar) bedekte mynuitskot, en 'n rots stortingsterrein wat voorheen bedek is (10-12 jaar gelede). Vyf rhizosfeer monsters van die volgende is ingesamel: *Cenchrus ciliaris, Enneapogon cenchroides, Tephrosia polystachya,* (plaaslike volop forb spesies) by terrein 1 en *Cenchrus ciliaris, Stipagrostis hirtigluma, Tephrosia polystachya*, en *Pennisetum setaceum* monsters by terrein 2. Die monsters van oop gebiede sonder plante is ook gemonster in beide terreine. Metagenomiese DNS is onttrek uit die versamelde monsters, hoewel verrykingstegnieke toegepas. Verdunnings reeks verspreiding plate is gebruik om kweekbare bakterieë wat teenwoordig is in die uitskot monsters te identifiseer. Polimerase ketting reaksies was geïmplementeer om amplikons te produseer van konserveerde streke in genomiese DNS van AMS en bakterieë wat teenwoordig in die mynskot. Die oorheersende genera van bakterieë bespeur in die versamelde uitskot monsters behoort aan *Bacillus*¸ maar as gevolg van die verryking tegnieke wat gebruik is kan dit egter nie beslis verklaar word dat hierdie bakterie die volopste in die omgewing is vanwaar die monsters versamel is nie.

As gevolg van die klein skaalse *ex situ* aard van die eksperimente, is die ooreenstemmende resultate wat verkry is uit hulle behandeling proewe en mikrobiese DNS isolasie nie heeltemal verteenwoordigend van die aard van die ekologiese omgewing teenwoordig *in situ*. Nietemin, PGBV behandeling op die gekose grasse het geen duidelike voordeel ontlok in gemeet groei parameters gebruik word vir doeleindes phytostabilisation. Trypan bevlekkkings

prosedures het aangedui dat meeste van die grasspesies in staat is om simbiotiese verhoudings met AMS te vorm, terwyl proewe aangedui het dat AMS plante kan baat vat teenwoordig is in die mynuitskot.

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Abbreviations

%	Percent
°C	Degrees Celsius
µg	Microgram
µg/mL	Microgram per millilitre
µL	Microlitre
µm	Micrometre
AMF	Arbuscular mycorrhizae fungi
ANOVA	Analysis of variance
BLAST	Basic local alignment search tool
bp	Base pair
ca.	Approximately
CAF	Central Analytical Facility
CBL	CropbioLife™
cm	Centimetre
ddH_2O	De-ionised distilled water
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
e.g.	For example
EtOH	Ethanol
eV	Electron volt
gDNA	Genomic deoxyribonucleic acid
i.e.	that is

IPB	Institute for Plant Biotechnology
L	Litre
LB	Luria-Bertani (media)
LC	Lumichrome
m	Metre
M	Molar
mg	Milligram
mg/L	Milligram per litre
mL	Millilitre
mM	Millimolar
m/v	mass/volume
NCBI	National Centre for Biotechnology Information
ng	Nanogram
nm	Nanometre
No.	Number
OD	Optical density
PCR	Polymerase chain reaction
PGPB	Plant growth promoting bacteria
PGPS	Plant growth promoting substances
RNA	Ribonucleic acid
Rpm	Revolutions per minute
rRNA	Ribosomal RNA
s	Second

SDS	Sodium dodecyl sulfate
SL	Strigolactone
SM	Smoke-water
sp.	Species (singular)
spp.	Species (plural)
TAE	Tris-acetic acid-EDTA
TE	Tris-HCl EDTA
Tm	Melting temperature
U/µL	Units per microliter
UV	Ultraviolet
V	Volt
v/v	Volume per volume
xg	Relative gravitational force

1. General Introduction and Literature Review:

Industrial methods for extracting minerals from the earth have developed tremendously since ancient times. This results in drastic environmental changes. As mineral resources are often embedded deep below the surface they are not easily accessible, hence the required removal of soil and vegetation cover (Bradshaw, 1997), often in large volumes and over extensive areas. Landscapes are left scarred and barren, with tailings, or mine dumps, that contain large amounts of harmful pollutants and contaminants, which are typically unsuitable substrates for plant growth. Recent global shifts towards environmental responsibility have led to techniques to combat the waste and degraded landscapes generated by mining processes. Heavy metals present at high concentrations in soil result in harmful consequences in the ecosystem. These substances could enter the food chain through agricultural products or contaminate water resources, thereby posing a serious threat to human health. However, some of the techniques employed to remedy contaminated sites are expensive and intrusive to the ecosystem. Therefore, rehabilitation of affected areas is considered important to ensure sustainable mineral extraction procedures. Common methods for rehabilitation include soil amelioration, soil washing, bioremediation, and phytoremediation (Figure 1.1) which encompasses phytostabilisation (*in situ* physical stabilisation of metal contaminants and tailings through root binding to soil particles to prevent erosion and leaching of harmful contaminants) and technologies such as phytoextraction (import and storage of heavy metals in plants' above-ground tissues), for treating metal-contamination (Leung *et al*., 2013). Poor soil fertility is often a leading constraint in the revegetation (process of replanting and rebuilding the soil of disturbed land) of active mining and mined out areas (Domingo & David, 2014). Soil amelioration is a strategy applied to improve soils to a point where they provide the necessary environment to sustain plant growth (Dexter, 1991). Phytoremediation involves the use of plants to transfer, stabilize, remove or destroy inorganic and/or organic contaminants present in contaminated soil (Vishnoi and Srivastava, 2008). This process utilizes plants to partially or fully remediate selected contaminants present. By the application of numerous plant biological processes and physical characteristics of plants to assist in site remediation, harmful contaminants such as chlorinated solvents, heavy metals, pentachlorophenol (PCP), petroleum hydrocarbons and polycyclic aromatic hydrocarbons (PAHs) can be removed (Vishnoi and Srivastava, 2008). Phytoremediation is therefore considered a multistep process that encompass different methods that result in contaminant degradation, removal or immobilization (Vishnoi and Srivastava, 2008), soil stabilisation, and ultimately revegetation.

Mine tailings are the result of the materials that remain after the extraction or beneficiation of ores. Natural revegetation of these sites is generally prevented by an amalgamation of factors beginning with metal toxicity (Mendez and Maier, 2008). Tailings are characterised by heightened metal concentrations such as copper (Cu), cadmium (Cd), manganese (Mn), arsenic (As), lead (Pb), and zinc (Zn) (1-50 $g.kg^{-1}$) (Bouiet and Larocque, 1998; Mendez and Maier, 2008). Tailings also lack organic matter and micronutrients, and typically display acidic pH, though some tailings are alkaline (Krzaklewski and Pietrzykowski, 2002). Due to these reasons, tailings do not

possess a normal soil structure and typically support a community of severely stressed heterotrophic microbes (Mendez *et al*., 2007). These microbial communities have exceptionally low species richness compared to uncontaminated soil, and display limited carbon utilisation diversity (Moynahan *et al*., 2002). Additionally, the microbial community of the tailings is dominated by autotrophic sulfur- and iron-oxidizing bacteria which are typically connected with plant death at acidic tailings (Schippers *et al*., 2000). Plant establishment in arid and semi-arid regions is additionally impeded by numerous physicochemical factors that include extreme temperatures, particularly at the surface of the tailing, truncated precipitation and high winds (Mendez and Maier, 2008). Mine waste disposal has traditionally involved the return of the waste materials to the mining site; dumping into a stream, lake, or ocean; or dumping them into a receiving pond. Presently, the most commonly used approach remains the containment of surface tailings within embankments (Mendez and Maier, 2008). An alternative strategy involves returning the tailing materials to the mine (back filling or in-pit storage) or mixing it with coarse mine waste (co-disposal). Dry-stacking facilities are commonly utilised in arid and semi-arid regions, whereby tailings are dried, spread out, and compressed (Mendez and Maier, 2008). However, these tailings continue to be unstable, unvegetated, and subject to water erosion and aeolian (wind) dispersion where there is the possiblility to contaminate surrounding communities and sensitive areas in the environment (González and González-Chávez, 2006).

Heavy metal contaminants are often created as a by-product of human industrial activities such as mining and smelting processes. Heavy metals can be grouped into a single category consisting of 53 elements with a specific weight higher than 5g/cm^3 (Holleman & Wiberg, 1985; Weast 1984). Some of these heavy metals, although toxic at various concentrations, have important functions in the physiological process of biological organisms. Trace elements such as copper, zinc, iron (Fe), nickel (Ni), and manganese are necessary for the regular development and growth of plants. Additionally, these elements are necessary for various enzyme-catalysed, electron transfer, redox reactions and have a structural function in nucleic acid metabolism (Cobbett, 2000). There are also non-essential metals such as cadmium (Cd), palladium (Pd), mercury (Hg), and arsenic (Mertz, 1981).

In terrestrial plants the roots are normally the organ in direct contact with metal ions present in the soil. It is necessary for the plant to control the acquisition of compounds to avoid deficiency or excess, by distributing them in a manner which ensures homeostasis. Specific uptake systems are responsible for the acquisition of essential heavy metals, although, if present in high concentrations as is typical of contaminated substrates, they can also enter plant cells through non-specific transporters. Non-essential heavy metals are capable of entering the plant root through passive diffusion, as well as through using low-affinity metal transporters with a broad specificity (Hall and Williams, 2003). If not regulated, heavy metals present at high concentrations are capable of interfering with enzymatic activities through modification of protein structures by replacing vital elements, causing nutrient-deficiency symptoms. Plant cells are therefore vulnerable to heavy metal toxicity, becoming functionally impaired by modifications of important intrinsic membrane proteins such as the vital H^+-ATPases (Hall, 2002). Additionally elevated levels of heavy metals results in the generation of reactive oxygen species that cause oxidative damage

of plants (Schützendübel and Polle, 2002). Growth retardation, chlorosis, root browning, cell cycle arrest and other toxicity symptoms are observable as a consequence of heavy metal contamination with the result of poorly vegetated mine dumps that are slow to recover naturally and which are resistant to supporting plants introduced through rehabilitation efforts. Consequently some plants have developed methods to maintain ion homeostasis under increased heavy metal concentrations (Clemens, 2001). The methods employed in these roots rely on regulating the enrichment, acquisition, trafficking and detoxification of heavy metals within the cell at susceptible locations by circumventing the generation of physiologically intolerable heavy metal concentrations. Detoxification methods include the binding of heavy metals to the rhizodermal cell walls and/or the extracellular heavy metal-chelation by root exudates. Heavy metal concentrations in the cytosol are controlled by active plant efflux systems. Within the plant cells, chelating agents such as metallothioneins and phytochelatins are produced, which have a binding affinity for heavy metals. The subsequent complex that results, can be transferred from the cytoplasm across the tonoplast to be sequestered inside the vacuole (Hall, 2002). Additional plant cell organelles are also utilised in storage of heavy metals. In plants, iron is stored bound to ferritin inside the chloroplast. Since heavy metals are not biodegradable and pose the risk of entering the food chain, they pose a long-term threat for human health and the environment (Jarup, 2003). Conventional methods for remediation rely on excavation and translocation of heavy metal polluted soil whilst phytoremediation offers a sustainable and inexpensive on-site approach to soil remediation practices. Strategies that utilize plants for remediation purposes are phytostabilisation and phytoextraction. These processes tend to be slow, therefore improving the efficiency and stabilization of removing contaminants is an important goal. The process of phytostabilisation relies on contaminant containment through field application of plants such as grasses growing on soil, sediment or sludges contaminating heavy metals (Leung *et al*., 2013). This produces a vegetative cap that is beneficial for long-term stabilisation and containment of the tailings. This vegetative canopy reduces aeolian dispersion and the plant roots are capable of preventing water erosion, and the immobilisation of metals by accumulation or absorption, and ensure a rhizosphere wherein metals can stabilise and precipitate. Phytostabilisation mainly focuses on the sequestration of metals present in the rhizosphere but not in plant tissues, unlike phytoextraction, or the hyperaccumulation of metals into root/shoot tissues of plants (Ernst, 2005). The major disadvantages in the application of phytoextraction by utilising naturally-occurring metal hyperaccumulators for continual extraction, are their slow growth rates, comparatively low biomass, and the absence of hyperaccumulators for the most environmentally important metallic contaminants. This often results in long time periods required for clean-up (Haslmayr *et al*., 2014).

As a result of phytostabilisation, metals concentrations subside and wildlife, livestock, and human exposure is limited (Wong, 2003). Phytostabilisation of mine tailings use salt-, drought-, and metal-tolerant plants to immobilise heavy metals in the tailings substrate present in arid and semi-arid environments. The bioavailability of metals and therefore toxicity will diminish as plants aid the metal precipitation to more insoluble forms. The occurrence of plants in mine tailings improves the heterotrophic microbial community, which could consequently stimulate plant growth and contribute in metal stabilisation (Mendez *et al*., 2007). The main objective of effective

phytostabilisation is the lasting succession of plant communities at mine tailings to stimulate microbial diversity, soil development processes and restore soil ecosystems to a state of self-sustainability.

Additionally, the direct targeting of the physical condition of the soil for rehabilitation by the introduction of plant species provides a beneficial role in stabilization through the process of soil amelioration. Soil can deteriorate from a number of causes such as human industrial activities or natural catastrophes. Soil stability can be remedied through binding together of soil particles by plant roots and fungal hyphae (Dexter, 1991). Plant roots are of particular importance, as they create biopores following their decay. Biopores comprise of the root channels and earthworm networks, which provide pathways for root penetration and subsequent plant growth. Plant roots play a vital role in soil as the root tip penetrates the soil without a pre-existing macro-structure, and subsequently produces a biopore, usually within a year for the non-lignified tissue of annual species. When biopores are produced much deeper in the soil profile, they last much longer than those produced near surface level. Wind, rain vehicular and animal traffic combine to collapse or fill up these biopore tunnels near the surface (Horn and Dexter, 1989).

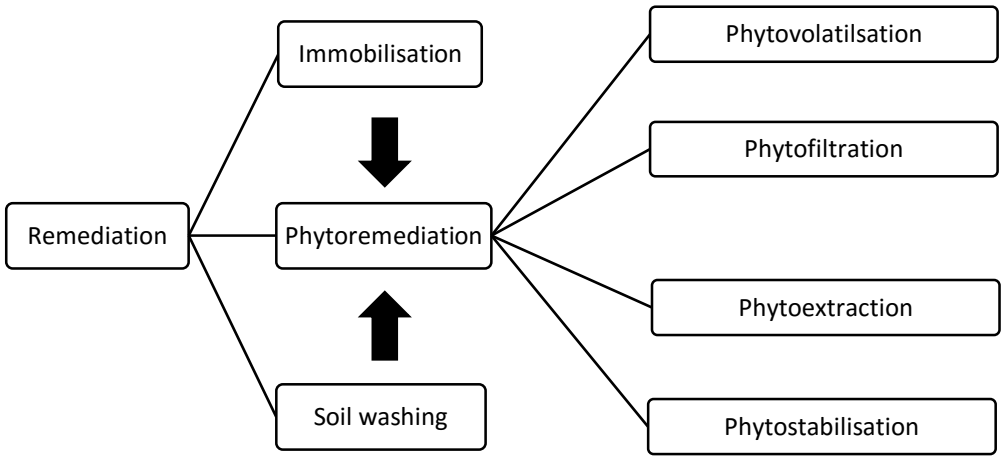

Figure 1.1: Common remediation approaches for heavy metal contaminants in soil. Adapted from Hao *et al*., (2014).

1.1 Arbuscular Mycorrhizal Fungi

Plants are often capable of having a symbiotic relationship with the microflora in the rhizosphere (Smith and Read, 2008). Arbuscular mycorrhizal fungi (AMF) are fungal symbionts which occur widely most ecosystems soils, including polluted soils. They have a global effect on carbon and phosphate cycling. These fungi are able to acquire and deliver a portion of the acquired nutrients to their host, thereby enhancing the nutritional state of their host. The plants subsequently provide the fungi with synthesized sugars. This evolutionary relationship is believed to

have evolved approximately 400 million years ago (Remy *et al*., 1994), consistent with the discovery of arbuscules in an Early Devonian land plant, *Aglaophyton major*. Arbuscular mycorrhizal fungi are presently comprised of three families; Glomaceae (*Glomus* and *Sclerocystis*), Gigasporaceae (*Gigaspora* and *Scutellospora*) and Acaulosporaceae (*Acaulospora* and *Entrophospora*) (Morton and Benny, 1990; Morton and Bentivenga, 1994). The most ancient family of AMF is Glomaceae; the Acaulosporaceae and Gigasporaceae appear to have evolved at a later stage and separated from each other in the late Paleozoic period, approximately 250 million years ago (Simon *et al*., 1993). Due to the ubiquitous nature of the symbiotic relationship and the fact that all AMF inhabit similar plant/soil niches, it is generally assumed that all the AMF species have the same function in their symbiotic state (Dodd *et al*., 2000). The symbiosis between fungi of the phylum Glomeromycota and plants is widespread. This relationship is critical for plant functioning given the vast majority of plant species that are dependent on it for nutrient uptake (Redecker and Raab, 2006). This task is efficiently accomplished by fungal symbionts through their extensive extraradical mycelium. Within the root cells of plants AMF form typical tree-like structures known as arbuscules, or hyphal coils. Some of these fungi are also capable of producing storage vesicles. Relatively large (40-800µm) spores with layered walls that contain several hundred to thousands of nuclei are produced by Glomeromycotan fungi (Becard and Pfeffer 1993). The phylum Glomeromycota encompasses approximately 200 morphospecies that have been described and have conventionally been identified by features of the spore wall. The method in which the spore is formed on the hypha ("mode of spore formation") has been a key factor to define families and genera, whilst the layered structure of the spore wall is utilised to differentiate between species (Morton, 1988). Most arbuscular mycorrhizal fungi were placed in the genus *Endogone* up until Gerdemann and Trappe (1974) divided them into four different genera in the order Endogonales (Gerdemann and Trappe, 1974). A new order "Glomales" was established in the Zygomycota by Morton and Benny (1990), which is comprised of six genera. Since then, further evidence have been collected that supports the view that arbuscular mycorrhizal fungi are distinct from other Zygomycota. Glomales do not appear to form the distinctive zygospores, and in all instances when the nutritional mode has been revealed they form mutualistic symbioses. Arbuscular mycorrhizal fungi are a sister group of Asco- and Basidiomycota based on their rDNA phylogeny and are not monophyletic with any part of the Zygomycota. Thus, the "Glomales" was elevated to the rank of a phylum Glomeromycota (Schüβler *et al*., 2001). In the same investigation, the order name "Glomales" was changed to "Glomerales" and several new orders were established.

1.1.1 Arbuscular mycorrhizal fungi in phytoremediation

As arbuscular mycorrhizal fungi are obligate symbionts, it is imperative that they form symbiotic relationships with plant hosts in any environment in which they occur. The benefit of this mutualistic relationship is paramount in sites considered nutrient deficient. These areas are often affected with contaminants, such as the presence of pollutants, heavy metals and industrial runoff. An estimated 95% of the world's plant species form symbiotic relationships with AMF (Smith and Read, 2008). These plant-fungi symbioses occur in almost all climates and habitats, and disturbed soils (Estaún *et al*., 1997), including those derived from mining activities (Weiersbye *et*

al., 1999). The investigation and application of AMF and plants symbionts specifically suited to remediation of contaminated areas is therefore of benefit. AMF symbiosis extends the plant root system due to the hyphal network functionally increasing the surface area available to the plant to absorb and transport nutrients (Gohre and Paszkowski, 2014). AMF can contribute to metal immobilization within the soil and physically stabilise soils by binding to soil particles (phytostabilisation), and additionally mycorrhizal plants are capable of showing enhanced heavy metal uptake and root-to-shoot transport (phytoextraction) of heavy metals. The mycorrhizal colonization during the removal of contaminated soils is dependent on the plant-fungus-heavy metal combination.

Arbuscular mycorrhizal fungi are among those soil microorganisms that offer a direct association between the soil and plant roots through the interaction with their hosts to form a symbiotic relationship in contaminated areas (Leung *et al*., 2007). Hence, they play a great role regulating toxic metal exposure and toxicology in plants. Research into the interactions between AMF and hyperaccumulators and non-accumulators and the potential role of mycorrhizae to aid in the survival of plants at toxic metal-contaminated soils is being investigated to understand ways in which mycorrhizal fungi can aid plants utilized for the revegetation of derelict land at different temporal and spatial scales.

It is important to improve the efficiency and increase the stabilisation level or removal of toxic metals from soils by plants. Greater attention is being paid to the role that fungi play in plants grown at metal-contaminated sites that possess poor nutrients, adverse physical conditions and low water-holding capacity (Vosátka *et al*., 2006). Evidence has been found that AMF plays a factor in increasing the tolerance of some plants to toxic metal contamination through developing metal tolerance in the fungi themselves and binding the metals to polyphosphates found within the fungal hyphae (Barea *et al*., 2005). The establishment of the mycorrhizal network offers several advantages to the host plant for the attainment of mineral nutrients: i) the hyphae of the fungi extends beyond the nutrient depletion area that surrounds the plant's roots, ii) the hyphae increase the available surface area for the absorption of nutrients, iii) the hyphae are capable of spreading into soil pores that are otherwise too small for the plant roots to enter and iv) some AMF have access to forms of phosphorus (P) and nitrogen (N) that might not accessible to non-mycorrhizal plants, specifically the organic forms of these nutrients (Morgan *et al*., 2005). Mycorrhizal symbiosis also conveys protection to the roots from metal toxicity by mediating interactions between plant roots and metals; the ability to moderate metal toxicity through mycorrhizal association in higher plants has been demonstrated in AMF (Leung *et al*., 2010b). Mycorrhizal fungi can directly protect plants from the build-up of phytotoxic concentrations of certain pollutants by secreting detoxifying compounds (e.g. organic acids) or by binding the pollutants into fungal tissues that are associated with the roots, thereby creating a physical barrier against toxic metal translocation to the plant itself (Vosátka *et al*., 2006). In phytostabilisation, the main role that AMF symbiosis provides is a favourable micro-environment that ultimately lets plant roots survive higher toxic metal concentrations, possibly through enriching the level of toxic metal at or in fungal structures. Hyphal binding is a vital sink for toxic metals due to the large surface area to volume ratio presented by the fungi in the soil. Additionally toxic metal-tolerant fungi possess a greater capacity to bind to toxic metals (Joner *et al*., 2000),

and therefore are very suitable for stabilising toxic metals in the soil. Additionally, fungi are capable of enhancing plant resistance and facilitate phytostabilisation in harsh environments. Two AM fungal species (*Glomus mosseae* and *Glomus caledonium*) were investigated by Gonzalez-Chavez *et al*. (2002), who isolated these AMF from an arsenate-resistant grass *Holcus lanatus* at a mine site. They discovered that these AMF (in *Holcus lanatus*) developed arsenate resistance, therefore conferring enhanced resistance to arsenic contamination of the host plant. The AMF that colonized *H. lanatus* were able to carry out their role by aiding the host to fix toxic metals within the rhizosphere zone, thereby preventing uptake of toxic metals into the plant.

1.2 Rhizobacteria

The role of numerous plant growth promoting bacteria (PGPB) regarding the efficiency of phytoremediation has been investigated. These bacteria play a role in the enhancement of plant resistance to metals, defence against plant pathogens, stimulation of plant growth, aid in nitrogen fixation, and improving phosphorus and nitrogen accessibility, phytohormone production (cytokinins and auxins) (Vivas *et al*., 2006), exudation of volatile compounds (2,3-butanediol and acetoin) (Ryu *et al*., 2003), organic acid and siderophores secretion (Ma *et al*., 2009), 1-aminocyclopropane-1-carboxylate deaminase (ACC) synthesis (Glick *et al*., 2007), as well as the accumulation and biosorption of metals (Chen *et al*., 2008). Rhizobia are a group of bacteria occurring in the soil that perform an important function in phytoremediation. These plant-associated bacteria migrate to the rhizosphere of living plants from the bulk soil to colonize the rhizosphere and plant roots (Kloepper and Schroth, 1978). The rhizobacteria such as *Arthrobacter*, *Achromobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Pseudomonas*, *Enterobacter*, and *Serratia* (Gray and Smith, 2005) as well as *Streptomyces* spp. have been discovered to have advantageous effects on numerous plants at metal-contaminated environments (Tokala *et al*., 2002; Dimkpa *et al*., 2008a; Dimkpa *et al*., 2008b). The exact mechanism via which plant growth stimulation occurs differs among bacterial strains, but most are dependent upon numerous metabolites released by the microbes (Ma *et al*., 2011). The role of rhizobia in the rhizosphere for bioremediation is outlined in Figure 1.2. The symbiosis between legume-rhizobia has traditionally been applied in agricultural practice to yield nitrogen to the plant and therefore enhance plant growth and is one of the beneficial plant-microbe interactions (De Hoff and Hirsch, 2003). Currently, this particular symbiotic relationship has been suggested for application in metal contaminated soil to ameliorate soil fertility and simultaneously remove or stabilise metals (Dary *et al*., 2010). Rhizobia are capable of directly enhancing phytoremediation through production of the plant growth promoting factors as previously mentioned and nitrogen fixation. Consequently, this would result in increased metal uptake and translocation from the soil to the plant due to the change in bioavailability. Additionally, microbial metabolism such as enzyme activities and extracellular polymeric substances (EPS) production can alter the redox state and/or immobilise metals to diminish their toxicity to plants (Hao *et al*., 2014). A number of *Rhizobium* strains are capable of absorbing and accumulating metals, thereby indirectly assisting phytostabilisation. Despite the legume-rhizobia symbiosis being especially sensitive to some metals, such as Cd (Zhengwei *et al*., 2005), which interferes with physiological

processes, there have been strains of *Rhizobium* isolated from soils of metal-contamination that have a resistance to some metals (Hao *et al*., 2014). Hence due to the role of rhizobia in promoting plant growth and health and ameliorating metal toxicity, extensive research should be undertaken to explore microbial distribution, diversity, and function in soil allochthonous (foreign) and autochthonous (indigenous) habitats.

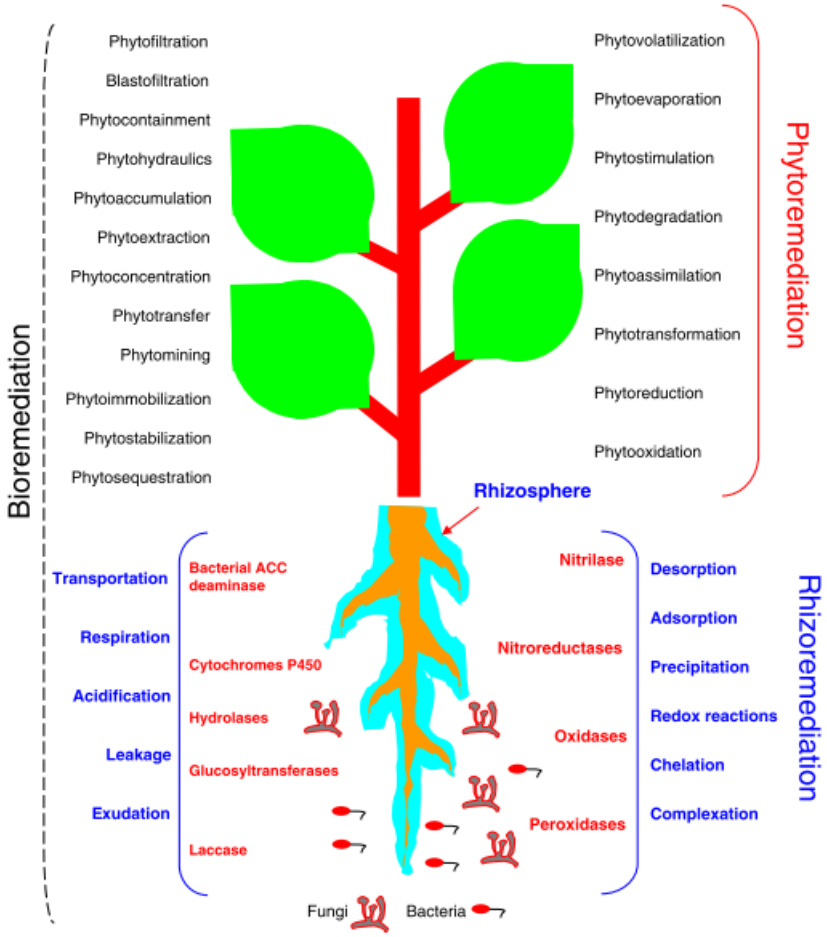

Figure 1.2: The importance and site of action of soil-plant microbial interactions for the bioremediation of metals and organics (pesticides, solvents, explosives, crude oil, polyaromatic hydrocarbons) (Ma *et al*., 2011).

1.3 Plant Growth Promoting Substances

Improving the survivability of plants at heavy metal contaminated sites is paramount in the phytoremediation of areas, particularly those with poor soil nutrition. The application of plant growth promoting substances (PGPS) to aid plant survival offers a direct and precise method to improve plant growth and health. These substances have the ability to directly and positively influence plant physiology, health, germination and the plant rhizosphere (Table 1.1). These substances have been used predominantly in laboratory experiments or applied to crops, but unlike AMF, which have been extensively studied for their role in assisting plant survivability in the phytoremediation of deleterious environments (Khan, 2005; Straker *et al*., 2007; Straker *et al*., 2008; Ortega-

Larrocea *et al*., 2010; Orłowska *et al*., 2011; Bhaduri and Fulekar, 2012; Cicatelli *et al*., 2014), the role of some of the PGPS in phytostabilisation has yet to be elucidated. The application of smoke-water to enhance the phytoremediation potential of *Pennisetum clandestinum* at cadmium contaminated sites has been investigated (Okem *et al*., 2015).

Table 1.1: Plant growth promoting substances (PGPS) studied and their effect on plant growth.

PGPS	Effect on Plant Growth	Reference
Strigolactones	Promote seed germination, apical dominance control, root elongation, root-hair elongation, promotes fungal symbiosis	(Koltai *et al*., 2010)
Lumichrome	Increase root biomass, stimulate seedling development, increase in biomass	(Gouws, 2009)
Smoke-water	Plant growth and development, seed germination, seedling vigour, flowering and rooting	(Jain and Van Staden, 2006; Gouws, 2009)
CropbioLife™	Protection from wilt disease, increases fruit yield, increased root exudation, promotes rhizobia symbiosis	(Dr N Hanekom, Pers. Comm. 2015).

1.3.1 Strigolactones:

The role and function of specific hormone groups involved in branching pattern control in plants should be considered in phytostabilisation purposes due to their role in influencing plant root branching and elongation. Branching patterns of higher plant species are intrinsic in nature. Environmental factors such as plant crowding, light intensity, nutrient limitation and insects are capable of inhibiting or stimulating branching, and therefore, plant architecture. A newly discovered hormone class is believed to play an extensive and pivotal role in regulating branching, and is involved in the mechanism that plants utilize to detect environmental signals and channel them towards growth or no growth.

1.3.1.1 Discovery and Potential

Strigolactones are a group of carotenoid-derived sesquiterpene lactones, first isolated 40 years ago by Cook *et al*. (1966) who reported the isolation of (+)-strigol from the exudates of cotton roots (*Gossypium hirsutum*). They were characterized as germination stimulants of parasitic weed seeds. Thus, originally these endogenously-produced compounds were regarded as detrimental to the producing plant (Cook *et al*., 1966). When the plant roots release these compounds, they trigger seed germination of root parasitic angiosperms such as witchweeds (*Striga* spp.) and broomrapes (*Orobanche* and *Phelipanche* spp.). These parasitic plants belong to the Orobanchaceae family and are the two most prevailing root parasitic plants, causing tremendous global crop losses. The seeds of these plants do not germinate unless they are exposed to strigolactones (Yoneyama *et al*., 2010). These plants are obligate parasites as their photosynthesis is not capable of supporting their survival unless they are connected to a host's roots, whereby they can exploit the host as a source of nutrients, water and assimilates. *Striga* species are specifically important due to their parasitism of essential food crops in Sub-Saharan Africa, the

Middle East, and Asia. Areas that are heavily infested often result in complete losses of harvests. These parasitic plants have extremely small seeds (0.2-0.4 mm) that are composed of relatively small number of cells (Joel *et al*. 1995). The survival of *Striga* and *Orobanche* are improved by their ability to produce up to half a million seeds that can remain viable in the soil for many years. The dormant long-lived seeds ensure that the root parasites are capable of adapting to changes in host availability, thereby make them difficult to control (Joel *et al*., 1995, Joel *et al*., 2007)

1.3.1.2 Function of Strigolactones

The function of strigolactones as a plant hormone was independently discovered by two different teams (Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008). Both identified strigolactones and their derivatives as endogenous plant hormones responsible for shoot branching inhibition. Branching control was primarily believed to be associated with cytokinin and auxin until discoveries of mutants in *Arabidopsis*, rice, pea and petunia indicated the existence of a factor that had a strong effect on bud outgrowth that was independent of known phytohormones (Goulet and Klee, 2010). Strigolactones as phytohormones that are exudated from plant roots have an additional role as a rhizosphere signal molecule that promotes hyphal branching of symbiotic fungi. This was discovered by the identification and isolation of 5-deoxy-stigol from the legumes *Lotus japonicus*, which was shown to induce hyphal branching in AMF. Currently this hormone group consists of 15 different members that have been structurally characterized, all sharing a common C_{19} structure (Figure 1.3) that consists of a tricyclic lactone (A-, B- and C-rings) that is connected through an enol ether bridge to a second lactone (D ring)(Xie *et al*., 2010). The A- and B- rings are variable, whilst the C-D part is highly conserved. It is possible for the A-ring to contain a variety of oxygen functionalities at differing positions and the stereochemistry at the B-C junction and C-2' is also variable. The molecular shapes of strigolactone sterioisomers are dependent on the chirality of the respective stereogenic centres present in the molecules. The synthetic strigolactone molecule, GR24, has two enantiomers, one with the most 'natural' configuration which is the most active stereoisomer, whilst its mirror image is only marginally as active (Zwanenburg *et al*., 2009). Germination studies have shown that the C-D part of the structure is sufficient for inducing germination activity, therefore the bioactiphore resides in this part of the strigolactone molecule (Mangnus *et al*., 1992; Zwanenburg *et al*., 2009). It was revealed that the methyl group at C-4' of the D-ring is essential for bioactivity (Zwanenburg *et al*., 1994). Recently is was shown that the plant protein DWARF14 (D14) is a strigolactone receptor, and the D-ring moiety of strigolactone is essential for its recognition by D14 (Nakamura and Asami, 2014).

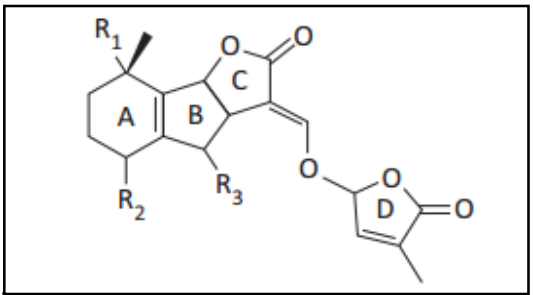

Figure 1.3: The general structure for strigolactones (Ruyter-Spira *et al*., 2013).

The enol ether conjugated with a carbonyl group of an ester (lactone) or ketone, connected to the D-ring was shown to be the minimal structural requirement for activity. The naturally occurring (+)-strigol is more active than

its derivatives, both natural and synthetic (Zwanenburg and Pospíšil, 2013). This class of plant hormones is derived from carotenoids through a pathway involving the iron-binding protein D27 and the carotenoid cleavage deoxygenase 7 and 8 enzymes (CCD7 and CCD8) (Alder *et al*., 2012). Strigolactones act as host-derived signals for rhizosphere communication of plants with AMF, where they act as an inducer of hyphal branching in the AMF (Akiyama *et al*., 2005). The symbiosis between plant and fungi was determined to be an ancient phenomenon that occurred millions of years ago. In phosphate-limiting conditions the synthesis of strigolactones is dramatically increased in plants (Yoneyama *et al*., 2007a; Yoneyama *et al*., 2007b) which results in AMF aiding the plant to increase phosphate uptake. This has the implication that strigolactones work as an integrator of nutrient signal and plant development (Umehara *et al*., 2008).

1.3.2 Lumichrome

Lumichrome is a product formed from the photo-degradation of riboflavin, or can be synthesized by bacteria (Gouws, 2009). Rhizobacteria can beneficially influence growth promotion in plants through either a direct or indirect way and can impact fundamental plant processes through the utilisation of signalling molecules. Phillips *et al*. (1999) were able to identify the molecule lumichrome from culture filtrates, as a rhizosphere signalling molecule that is capable of promoting plant growth. Riboflavin is converted to lumichrome through the photochemical-induced cleavage of the ribityl groups in the presence of light under acidic or neutral conditions as demonstrated by Yagi (1962). *Pseudomonas*, are capable of enzymatically converting riboflavin to lumichrome (Figure 1.4), and therefore light is not always necessary for conversion in the natural rhizosphere environment (Yanagita and Foster, 1956). Consequently, the role of lumichrome is frequently associated with riboflavin (Yanagita and Foster, 1956). The compound lumichrome is found in biological material in association with flavins and can contribute in biological processes. When lumichrome is applied it acts as a photosensitizer whereby it produces an oxygen singlet under light exposure. Experiments have indicated the beneficial role that lumichrome plays on plant growth. Research have reported major developmental changes that are generated by lumichrome at a very low nanomolar concentration (5 nM) in plants, which comprises of early initiation of trifoliate leaves, expansion of trifoliate and unifoliate leaves, greater biomass accumulation in monocotyledonous and dicotyledonous species, increased leaf area and stem elongation (Dakora *et al*., 2015). Additionally, lumichrome was shown to positively influence photosynthetic rates, increase root respiration, and changes stomatal conductance and transpiration (Volpin and Phillips, 1998; Phillips *et al*., 1999; Joseph and Phillips, 2003; Matiru and Dakora, 2005a; Khan *et al*., 2008). The promotion of growth effects are not specific to

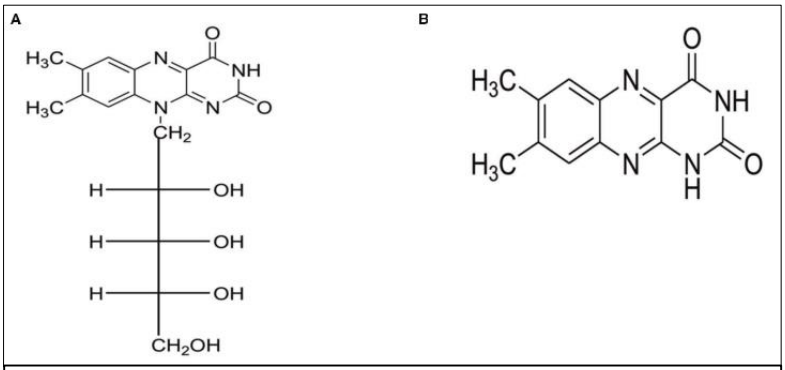

Figure 1.4: Chemical structure of A) Riboflavin and B) lumichrome (Dakora *et al*., 2015).

age, but these responses do vary between plant species. Importantly, the signal molecule's presence in high concentrations in the rhizosphere had inhibitory effects on plant growth (Matiru and Dakora, 2005b).

1.3.3 Smoke-water

Wildfires have been noted to stimulate the germination of quiescent seeds, and release nutrients stored in the plant tissues. This is viewed as an important environmental cue and germination promoter of seeds for various plant species (Nelson *et al.*, 2011). Recent research has shown that smoke generated by wildfire have the ability to stimulate germination and improve seedling vigour (Kulkarni *et al*., 2007). The method of action regarding the effect on seed germination by fire was observed in plant species that have particularly hard, water-impermeable seed coats. Fires are capable of cracking the seed coating to enable ensuing water uptake and initiate germination. Recently, a more practical and convenient method for triggering seed germination, instead of heat application was discovered. This method allows for smoke or aqueous extracts of smoke to function as a stimulant of germination (De Lange and Boucher, 1990). Smoke-Water (SM) is produced by bubbling smoke through a container of distilled water. The application of this solution has been shown to be very effective in stimulating seed germination in responsive species (Light *et al*., 2010). The key bioactive signal in smoke was discovered to be a butenolide 3-methyl-2H-furo[2,3-c]pyran-2-one, now known as karrikinolide, or KAR_1 (Flematti *et al*., 2004; Van Staden *et al*., 2004). This discovery revealed a class of plant bioactive molecules that are presently referred to as karrikins (Nelson *et al*., 2009). It has subsequently been shown that karrikinolide improves germination and seedling vigour of several species, including species from environments that are prone to fire (Light *et al*., 2009). Additionally there are structural parallels between the butenolide compound and the naturally occurring germination stimulant, (+)-strigol; the B-ring of KAR_1 and the D-ring of strigolactones are identical (Figure 1.5). Since their identification in smoke, karrikins have been tested on a substantial number of plant species for germination response, including not only plant species from fire-prone environments, but also popular crop plans such as tomato, okra, lettuce, rice, bean, and maize (Van Staden *et al*., 2004; Jain and Van Staden, 2006; Kulkarni *et al*., 2006; Van Staden *et al*., 2006). In comparison with crude smoke extracts, karrikins are able to affect a broad range of species, have a broader active range of concentrations and do not prevent seed germination as observed with increased concentrations of crude smoke solutions (Flematti *et al*., 2004; Van Staden *et al*., 2004). In addition to germination stimulation, seedling growth was also shown to be improved (Papenfus *et al*., 2015). However, pure karrikins are not available commercially and research-based applications may require large amounts of the pure compound. Hence the application of smoke-water offers an inexpensive environmentally-friendly alternative in phytoremediation to promote and assist in germination of plants involved in rehabilitation of heavy metal contaminated soil (Coons *et al*., 2014). The application of smoke-water in phytoextraction at cadmium-contaminated mine tailings have been

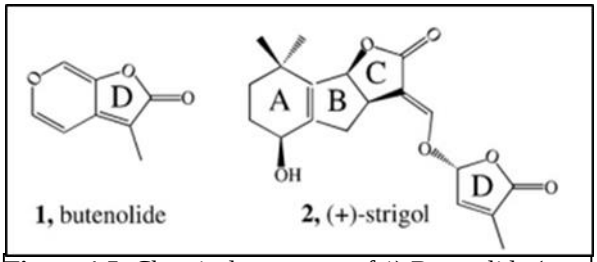

Figure 1.5: Chemical structures of 1) Butenolide (van Staden *et al*., 2004) and 2) (+)-strigol (Mangnus and Zwanenburg, 1992).

investigated and shown to enhance phytoextraction potential (Okem *et al*., 2015), by improving seedling vigour in *Pennisetum clandestinum* (Okem *et al*., 2015).

1.3.4 Flavonoids

CropbioLife™ (CBL) is a commercially available nutrient synergist that is a natural foliar flavonoid application, derived from various plant extracts (Biorevolution.co.za, 2015). The application of CropbioLife™ has shown an increase in harvest yield and plant growth and stimulates resistance to insect and detrimental bacterial and fungal agents. It also serves as a preventative measure for aphid infection through the addition of a layer of flavonoids to protect the phloem sap from aphid infestation (Dr N Hanekom, Pers. Comm. 2015). Flavonoids are secondary plant metabolites that have important physiological functions (Taylor and Grotewold, 2005). These functions range from plant pigmentation to auxin transport inhibitors and antioxidants (Winkel-Shirley, 2001), as well as signalling to symbiotic microorganisms (Wasson *et al*., 2006). Numerous plant species utilize flavonoids as signals and defence compounds in their interactions with pathogenic and beneficial microbes. The roots of legumes are capable of exuding specific flavonoids into the immediate soil that acts as chemotactic attraction signals for nitrogen-fixing symbiotic bacteria. These flavonoids are also responsible for the activation of *nod* genes expression in these rhizobia (Djordjevic *et al*., 1987), which are responsible for the synthesis of bacterial signals, Nod factors, which are essential for the initiation of a new plant organ, the nodule (Dénarié and Cullimore, 1993).

Flavonoids also produce a physical barrier to pathogen infection through promoting the lignification of the cell wall and it functions as an antioxidant by scavenging reactive oxygen species (ROS) which may result from stress or pathogen attack within the plant (Agati *et al*., 2012). When CropbioLife™ is applied, the flavonoids are concentrated in the periderm to assist the plant against wilt disease (a fungal infection) and other attacks. Additional phenolic compounds, together with CropbioLife™ are stored in these specialized cells, from where they are infused into attacked tissue such as the xylem vessels. The presence of CropbioLife™ can also alter tissue differentiation and cause the closure of vessels and block aggressive pathogens. The majority of trials utilizing CropbioLife™ have been on crop plants, which have shown various advantageous responses (Hendricks *et al*., 2015). When applied, CropbioLife™ induces plants to produce additional flavonoids leading to enhanced photosynthesis (Dr N Hanekom, Pers. Comm. 2015). Additionally, CropbioLife™ promotes symbiosis in the rhizosphere between plant and soil flora, resulting in an increased yield of root exudates.

1.4 Revegetation

Revegetation of mine tailings for phytostabilisation is one of the most common rehabilitation methods used globally. Identifying specific plant species, typically grasses, suited to facilitate the stabilisation or extraction of harmful contaminants at industrially active sites is paramount in the remediation process. Phytomanagement by phytostabilisation has been determined to be a suitable method to control erosion and metaloid-enriched leachates in metalliferous mine tailings (Robinson *et al*., 2009). This method capitalises on plants' ability to fix the soil and

immobilize metals within the rhizosphere at mine tailings where heavy metal enriched particles can spread through wind, water run-off and/leaching (Parraga-Aguado *et al*., 2013a). To ensure stabilisation occurs suitable plants must be able to grow at mine tailings under harsh edaphic conditions such as high metal concentrations, low pHs, low nutrient content, low water holding capacity, and additionally in semi-arid regions which harbour drought and salinity conditions (Conesa and Schulin, 2010). Local ecotypes that are capable of spontaneously colonising metal wastes are considered a useful tool to employ in phytostabilisation of mine tailings (Mendez and Maier, 2008). This usually comes at a cost to biodiversity as pioneer vegetation is generally constricted by the previously mentioned edaphic limitations, resulting in areas of low diversity of a few tolerant plant species which are capable of colonising these environments due to a lack of competitors (Macnair, 1987).

The phytostabilisation of mine tailings in semi-arid and arid environments requires the establishment of a diverse plant community through the inclusion of salt-, drought-, and metal-tolerant plants that cannot hyperaccumulate toxic metals into shoot tissues (Mendez and Maier, 2008). These candidate plants should ideally be native to the area, as they have survival mechanisms that have evolved to suit the severe climate of semi-arid and arid environments. Selecting various trees, shrubs, and perennial grasses for revegetation purposes is a crucial factor for phytostabilisation. Grasses temporarily limit aeolian dispersion of tailings by providing a quick ground cover, whilst trees and shrubs become established (Williams and Currey, 2002). Additionally, grasses and shrubs establish a deep root network that prevents long term erosion as well as providing a widespread canopy cover. Trees and shrubs provide grasses a high nutrient environment and reduce the moisture stress, as well as enhance the physical soil characteristics in semi-arid and arid climates (Belsky *et al*., 1989). Due to selective pressures, only a limited number of plants may ultimately dominate the ecosystem, the effect and presence of scarcer species is still significant to promote an ecosystem that is self-sustainable (Tilman *et al*., 2001).

These pioneering species often include weeds, grasses and opportunistic species that are able to create a "functionally limited" ecosystem with a low capacity of reaching a self-sustaining cycle (Parraga-Aguado *et al*., 2013b), and external factors such as short rainfall or drought may compromise their long-term sustainability. Therefore, the long-term goal in the restoration of a degraded site will be the establishment of sustainable plant communities that are able to mimic the surrounding flora diversity (Jefferson, 2004). Approaches recently have indicated the importance of utilising combinations or assemblages of species (Parraga-Aguado *et al*., 2013b). This ensures that the employment of species with varying ecological functionality will assure the long-term sustainability more efficiently than monospecific or limited species combinations. As an example, grasses may ensure fast-growing coverage, whilst trees support better soil protection against erosion (Parraga-Aguado *et al*., 2013a).

Plants typically utilised in phytostabilisation applications must be metallophytes (metal-tolerant plants), however, they should not accumulate metals or metal accumulation should be limited to root tissues. Metallophytes have evolved mechanisms to inhibit metal translocation in the above-ground plant mass, thereby preventing excessively

high metal concentrations in the shoot material (Mendez and Maier, 2008). Grasses best suited for this endeavour are chosen based on their perenniality and succession status and whether or not they are indigenous to the area. Pioneering plants are often selected based on their ability to grow in disturbed areas and colonize areas that are barren of plant life. Additionally, their grazing value could be considered for the added benefit of providing fodder for herbivores that might inhabit the area. Symbiotic relationships between plants and microbes in the rhizosphere provide plants with an increased probability of being effective for remediation purposes. *Cenchrus ciliaris* and *Cynodon dactylon* are grass species that have typically been utilized in South Africa, for rehabilitation and revegetation purposes (van Rensburg *et al*., 2003; Khan *et al*., 2007; Straker *et al*., 2007) due to their tolerance of heavy metals. Both grass species are indeed employed by Palabora Copper, as well three additional species *Anthephora pubescens*, *Chloris gayana* and *Panicum maximum*, which have been utilised in revegetation purposes elsewhere (van Rensburg *et al*., 2003; Rensburg and Morgenthal, 2004). The application of seed mixes promotes species diversity to minimise singular species dominance (Jefferson, 2004).

1.5 Study Site

Palabora Copper (PC) is the only producer of refined copper in South Africa, and provides the local market with 85% of its copper requirements (Palabora.com, 2015). Copper is the primary product of the company whilst also supplying by-products that include magnetite, nickel sulfate, anode slimes, sulfuric acid and vermiculite. The company operates one of the largest underground copper mines in the world, which started out as an open cast pit, but is now an extensive underground mining operation, and a significant amount of waste is generated. Significant amounts of sulfur dioxide are produced as waste during the smelting of copper. Sulfur dioxide is converted to sulfuric acid at an Acid Plant by the mine, where it is sold to numerous industries for the production of fertiliser. Magnetite is a mineral that occurs in the ore body, Palabora Copper washes and cleans some of the magnetite for the coal industry, where it is used in heavy medium separation processes. Nickel sulfate builds up as a waste material in electrolytic cells at the copper refinery, where it is collected, concentrated and sold to numerous industries. During the mineral extraction processes in the open pit, waste rock that was generated was dumped at a designated area and has since been rehabilitated and revegetated. Tailings produced from the mining concentrator are disposed of at one of two tailing impoundments. Magnetite tailings are stored in a dam for reprocessing, and copper tailings are disposed of at a copper tailings dam. Slag that is produced from the smelting processes is dumped at a designated area. These dumps are intended to be rehabilitated at the closure of the mine (van Dyk, 2015). The main rehabilitation strategy of Palabora Copper is revegetation of tailing dams and rock dumps, to provide plant cover to ensure minimal water and wind erosion at the surface, thereby the potential threat to the surrounding environment is reduced. Revegetation additionally assists to moderate the visual effect of the mined areas. The main rehabilitation strategy revolves around revegetating waste rock dumps and dams formed from terraces of approximately 5 m lifts (Surmon, 2015). These lifts are capped with vermiculite waste material, once they are completed to a depth of 25-30 cm. After capping is completed, the lifts are fertilised and seeded with a

commercial seed mix. Numerous tree species such as *Acacia* spp., *Combretum* spp., *Bolusanthus speciosus* and wild sand olives (*Dodonaea viscosa*) are also planted. Some of these, however, have a tendency to be pulled out by wild animals in the area such as baboons and elephants. When the grasses have germinated they are maintained and monitored to ensure that a suitable, self-sustaining cover of the lifts is achieved. A set of parameters are taken into account for revegetation such as; vigour, number of plants, species composition, and basal cover. This assists determining whether the revegetation program is succeeding and if there are changes that are necessary to be implemented (Surmon, 2015). Natural plant succession and colonisation of capped areas by local grass species also takes place (Surmon, 2015).

1.6 Aim

The aim of this research project was to investigate means of improving the phytostabilisation of tailings dumps generated by the copper mining activities at Palabora Copper. In order to achieve this, a number of objectives were set. Firstly, the grasses used by PC in their phytostabilisation program were investigated to determine their ability to germinate and establish in the vermiculite capping material used on the tailings dumps. A second, related aim, was to identify other pioneer grass species, particularly from species that establish naturally on the dumps, which could be included in the seed mix to enhance the efficacy of the seed sowing program. Thirdly, research was conducted to determine the effect that various plant growth promoting substances (PGPS) would have on the survivability and growth of grass species in deleterious environments, such as mine tailings, in the hope that these could improve vegetation recovery and so the efficiency of the phytostabilisation process. The grasses utilised for phytostabilisation purposes by Palabora Copper, as well as other potential species identified (objective 2), and their responses to PGPS treatments were investigated to determine if the treatments would aid in their survival. The PGPS treatments consisted of Lumichrome, a synthetic strigolactone (GR24), smoke-water, CropbioLife™, or arbuscular mycorrhizal fungi (Mycoroot™). A final objective was to investigate the microbial diversity present in the rhizosphere of selected plant species growing in tailings that were capped i) two or ii) 10-12 years ago, to gain an understanding of the soil microbiota in these tailings dumps. Due to the important role that, in particular, rhizosphere bacteria have on plant growth, and since the selective pressures present in the mine tailings will impact the diversity of the mine tailings, the possible presence of beneficial rhizosphere bacteria and fungi was investigated.

2. Materials and Methods

2.1 Reagents and Biological Materials

Molecular biology grade reagents were obtained from Sigma Aldrich Fluke (St. Louis, MO, USA), Merck (Wadeville, Gauteng, South Africa) and Promega (Madison, WI, USA), unless stated otherwise. Additionally, all nucleic acid modifying enzymes were obtained from Thermo Fisher Scientific (Pretoria, Gauteng, South Africa) and New England Biolabs® Inc. (Ipswich, MA, USA). Primers utilized in the study were synthesized by Inqaba Biotec™ (Inqaba Biotechnical Industries, South Africa).

Grass seed was acquired from Diverse Ecological Solutions (Pty). Ltd, (Pyramid, South Africa) or harvested *in situ* from the PC mine environment. Potting soil (Double Grow all-purpose organic potting soil, Nu Rust, Durbanville, South Africa) was utilized in germination trials, and commercial vermiculite (Rosarium Vermiculite, Lyndoch, South Africa) was used to grow grass seeds for use in treatment trials.

Mycoroot™ SuperGo arbuscular mycorrhizal fungal pellets were obtained from Mycoroot™ (Pty) Ltd, South Africa. CropbioLife™ (Biorevolution, Paarl, South Africa) was utilized as a foliar flavonoid in the application of grass treatment trials. Smoke-water, produced from burning fynbos material according to the method described in Baxter *et al*. (1994), was obtained as a 10^5 times concentrated solution from Kirstenbosch Botanic Gardens (Cape Town, South Africa). Lumichrome was obtained from Sigma Aldrich Fluka (St. Louis, MO, USA), and GR24 was acquired from Prof. B. Zwanenburg from the Department of Organic Chemistry, Radhoud University, Nijmegen (The Netherlands).

2.2 Site inspection and collection of study material

Palabora Copper is located in the central Lowveld of Limpopo, South Africa (24°0'8" S, 31°8'36" E), adjacent to the Kruger National Park. Unused capping material, which consists of unexfoliated vermiculite waste was used as the substrate for germination ad growth trials following treatments, was collected from the PC vermiculite dump site (23°57'59.53"S, 31° 9'31.08"E) in August 2014 and again in May 2015. Sampling of the rhizosphere of the most common locally occurring grass and forb species present on the rock dumps and mine tailings was performed by carefully digging out the relevant plants and collecting the soil immediately surrounding or attached to the roots. This material was vacuum sealed and stored at 4°C until analysis. Two sites were sampled; Site 1) A mine tailing site capped with 20-30 cm of vermiculite waste two years previously, and Site 2) A rock dump previously capped with vermiculite waste 10-12 years ago. At Site 1 (24° 0’27"S, 31° 11’48"E) a rhizosphere sample was collected from five individuals of each of the following; *C. ciliaris*, *Enneapogon cenchroides*, the forb *Tephorisia polystachya*, and an unvegetated ‘open’ area of at least 50 cm diameter. At Site 2 (24° 0’58"S, 31° 9’10"E) rhizosphere sampling included *C. ciliaris*, *Stipagrostis hirtigluma*, *Tephrosia polystachya*, *P. setaceum* and an ‘open’ area as defined earlier. See Figure 1.6 for the location of the source of capping material (vermiculite dump), as well as rhizosphere sampling sites (Sites 1 and 2) and seed collection areas (Sites A-C).

Figure 1.6: Location of sample sites at Palabora Copper. Site A = *Stipagrostis hirtigluma* and *Cenchrus ciliaris*, Site B = *Stipagrostis hirtigluma*, *Pennisetum setaceum*, and *Aristida adscensionis*, Site C = *Enneapogon cenchroides*, Site 1 = mine tailing, Site 2 = rock dump.

2.2.1 Grass Species Selection

Grass species were sourced from Diverse Ecological Solutions Pty. Ltd. These included the species in the commercial seed mix currently used by Palabora Copper *viz. C. ciliaris*, *A. pubescens*, *C. gayana*, *C. dactylon*, and *P. maximum*. This seed mix is widely utilised in rehabilitation and revegetation programs in the mining industry (van Rensburg *et al*., 2003; Rensburg and Morgenthal, 2004; Li, 2006; Granger and O'Connor, 2014). Additional grass species were chosen based on the following criteria: indigenous to the central Lowveld, their ability to grow in disturbed areas, perenniality, pioneering capacity, and ideally, rhizomatous and not tuft-forming habit (Van Oudtshoorn, 2012). These were as follows *Eragrostis teff*, *Paspalum notatum*, *Melinis repens*, *Stipagrostis uniplumis*, *Tragus berteronianus*, and *E. cenchroides*. In addition to these purchased seeds, seeds were collected *in situ* from locally-common grasses occurring naturally on site. Three sites were selected to harvest grass seeds. At Site A (24°0'52.02"S, 31°8'53.82"E) *S. hirtigluma* and *C. ciliaris* were collected, at Site B (24°0'54.32"S, 31°8'58.79"E) *S. hirtigluma*, *P. setaceum*, and *Aristida adscensionis* were harvested and at Site C (24°0'58.41"S, 31°9'9.07"E) *E. cenchroides* was collected. These sites were located on the south-east facing ridge

along the plateau of Site 2. Grass seeds were collected during May 2015 by hand and stored at room temperature in labelled paper bags. Parameters of grass species selection for revegetation and phytostabilisation are indicated in Table 1.2.

Table 1.2: Grasses selected for treatment response trials (Van Oudtshoorn, 2012), D.E.S = Diverse Ecological Solutions (Pty), PC = Palabora Copper.

Grasses	Sourced	Indigenous	Rhizomatous	Used by PC	Plant Status	Perennial / Annual	Disturbed areas occurrence
Anthephora pubescens	D. E. S	✓	×	✓	Climax grass	Perennial	✓
Cenchrus ciliaris	D. E. S	✓	×	✓	Subclimax	Perennial	✓
Chloris gayana	D. E. S	✓	×	✓	Subclimax	Perennial	✓
Cynodon dactylon	D. E. S	✓	✓	✓	Pioneer	Perennial	✓
Panicum maximum	D. E. S	✓	×	✓	Subclimax	Perennial	✓
Stipagrostis uniplumis	D. E. S	✓	×	×	Pioneer	Perennial	✓
Eragrostis teff	D. E. S	×	×	×	Pioneer	Annual	✓
Paspalum notatum	D. E. S	×	✓	×	Pioneer	Perennial	✓
Melinis repens	D. E. S	✓	×	×	Pioneer	Perennial	✓
Tragus berteronianus	D. E. S	✓	×	×	Pioneer	Annual	✓
Stipagrostis hirtigluma	*in situ*	✓	×	×	Pioneer	Perennial	✓
Aristida adscensionis	*in situ*	✓	×	×	Pioneer	Annual	✓
Enneapogon cenchroides	*in situ*	✓	×	×	Pioneer	Annual	✓
Pennisetum setaceum	*in situ*	×	×	×	Pioneer	Perennial	✓

2.3 Germination efficacy and PGPS response

2.3.1 Germination Trials

To determine the germinability of the seed lots of the selected grass germination trials were conducted. This was accomplished by placing 25 seeds per species on top of potting soil (Double Grow potting soil) in 25 cm diameter pots, with four replicates per species (n=100). A further trial was conducted using the waste vermiculite capping material as the germination medium. Seeds were covered with 5 g of their respective substrate material. Seedlings per pot were counted daily until no change in germination percentage was observed for a period of 7 days. Pots were individually watered with 20 mL of dH_2O daily via syringe application at noon.

Two pot trials were completed immediately outside the Institute of Plant Biotechnology (IPB) laboratory in an open area adjacent to the laboratory, and so were subject to ambient environmental conditions. As such seeds and seedlings experienced the sunlight hours, rain, wind, humidity, and temperature of Stellenbosch during the periods. The first trial was conducted over 43 days, after which no further germination was observed. This trial took place from 29th of January until the 15th March 2015, the repeat second trial from the 1st of April to the 16th of May 2015. The first trial received ±300 hours of cumulative sunshine, with average temperatures of 33°C, whereas the second trial received ±200 cumulative hours of sunshine and an average temperature of 26°C.The area where the trials were conducted received partial shade in the mornings and afternoons but full sun around midday.

A further set of trials was conducted in the greenhouse at the IPB, with plants receiving a controlled 16 hours of light and 8 hours of dark per 24-hour cycle. The first repeat was conducted over 43 days from 1st of June to 15th of July 2015. Greenhouse temperatures during this time ranged from 20-27°C. The second trial ran from the 20th of July until the 3rd of September, with temperatures ranging from 22-29°C. A grass seed was considered as having germinated when a sprout emerged from their substrate covering. Microsoft Excel was used to calculate percentage germination and the average germination rates of all studied grass.

2.3.2 Growth Trials and the Influence of PGPS

Treatment with the various growth promoters was applied to all grass species. For these trials, grass seeds were germinated in plastic trays filled with vermiculite (Rosarium Vermiculite CC). Trays were watered to capacity on the first day, and subsequently watered daily for two weeks. In order to ensure that any differences in growth were due to the treatments and not due to differences in age or initial size, two week old seedlings of the same size were selected and transplanted to 7.5 cm diameter pots containing waste vermiculite capping material from Palabora Copper. A single treatment trial consisted of three individual pots; with three seedlings per pot (n=9). Two trials were conducted outside the IPB laboratory under ambient conditions and coincident with the germination trials. Likewise, a further two trials were conducted inside the greenhouse, again coincident with and under the same conditions as the germination trials. Outside treatments consisted of a weekly syringe application of 10 mL of lumichrome (5 nM), GR24 (0.1 µM), CropbioLife™ (5 $mL.100L^{-1}$) or smoke-water ($1mL.100L^{-1}$). Control seedlings were supplied with 10 mL dH_2O. The greenhouse trials consisted of a weekly application at noon of 10 mL of lumichrome (5 nM), GR24 (0.1 µM), CropbioLife™ (5 $mL.100L^{-1}$), arbuscular mycorrhizal fungi (Mycoroot™). Non-treatment control samples received 10 mL dH_2O. Lumichrome stock solutions (1 mM) were prepared by weighing out 0.5 mg lumichrome in a 2 mL microfuge tube, then adding 1 mL of methanol and vortexing for 2 h. Forty µL of 1M of HCl was then added to the solution as well as 1960 µL of methanol, followed by shaking for an additional 2 h. To get the final solution, 50 nanolitres of the 1 mM lumichrome stock solution was diluted in 10 mL of ddH_2O. A 100 µM GR24 stock solution of was prepared by dissolving GR24 in 200 µL of acetone and then adding 800 µL of ddH_2O. The stock solution was diluted 1 000 times to prepare the final solution. For the Mycoroot™ AMF treatment, 0.09g of AMF pellets were crushed and thoroughly mixed into 180 g of vermiculite waste capping material per 7.5 cm pot, prior to transferring the seedlings to the pot. Outside trials were conducted over a period of 5 weeks, whilst inside trials lasted 4 weeks due to time constraints.

2.3.3 Growth Variables and Statistical Analyses

The effect of treatment on seedling growth was determined through measures of height, stem length, root length, number of leaves, fresh mass and dry mass. The height of the plant was defined as the total length of the longest leaf of the plant above the substrate surface, whilst stem length was the distance from the crown (area above ground of a plant) to the start of the leaf sheath. Seedlings were carefully extracted from their pots, gently rinsed with tap water and measured individually. The roots of AMF-treated plants were collected for Trypan-Blue staining to

confirm root colonisation by fungal hyphae. Dry mass of samples was measured following drying in an oven at 70°C for three days. Microsoft Excel was utilized to create graphs of treatment efficacy versus a specific variable of a tested grass species. Experimental data was analysed using STATISTICA Version 12 (StatSoft Inc. 2005), unless stated otherwise. One-way analysis of variance (ANOVA) was applied to determine the significance in differences between treatments. If the ANOVA test indicated a significance difference between treatments, Fisher's LSD Test was carried out as a *post-hoc* test. A *P*-value <0.05 was regarded as significant.

2.3.4 Trypan-Blue Staining

A modified protocol of Koske and Gemma (1989) was utilised to stain root material. Roots of grasses that received the AMF treatment were placed in 50 mL Erlenmeyer flasks, completely covered with 10 % (m/v) KOH, and chilled to 4°C for a week. All roots from a single pot were combined in a single flask. Thereafter the KOH was decanted, fresh KOH added, and the flasks autoclaved. Root tissue was then triple-rinsed under running water before being placed back in the flasks with enough 5 % HCl added to cover the sample. After 1 min the HCl was poured off, and sufficient Trypan-Blue stain (Thermo Scientific) was added to cover the samples, which were left overnight. The Trypan-Blue stain solution consisted of 1 L glycerol, 950 mL ddH_2O, 50 mL acetic acid, and 0.2g Trypan-Blue. Stained roots were then removed from their flasks, placed on a microscope slide with glycerol, and gently squashed using a coverslip before visualization using a light microscope. An OptixCam microscope camera was used to take pictures of the slides and viewed on TSview (version 7.3.1.7).

2.4 Rhizosphere Diversity

To identify the rhizosphere diversity two approaches were implemented; one consisted of metagenomic DNA extraction, to extract total microbial DNA present in collected samples. The second approach utilised dilution series spread plates to visually observe culturable microbes present in the collected rhizosphere samples from Site 1, to determine the presence of beneficial bacteria at a newly capped mine tailing. PCR was conducted on the isolated metagenomic DNA and colony PCRs from microbial colonies growing on the plates (Figure 1.7).

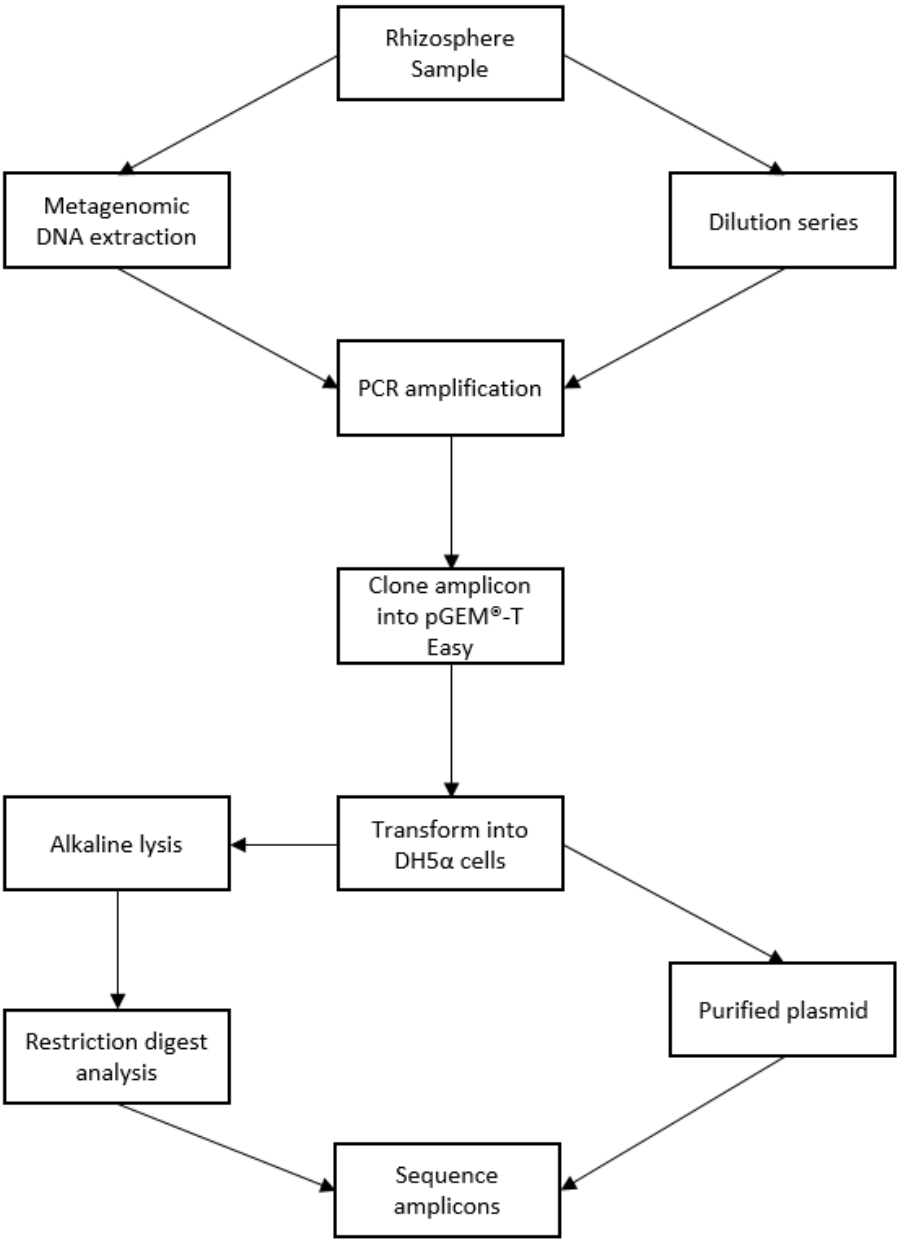

Figure 1.7*:* Methodology followed for microbial diversity analysis.

2.4.1 Metagenomic DNA Extraction

Soil DNA extractions were carried out on the collected rhizosphere samples using a ZR Soil Microbe DNA MiniPrep™ kit (Zymo Research). Extracted DNA was then quantified using a Thermo Scientific Nanodrop Lite spectrophotometer and then stored at -20°C until utilized for subsequent molecular analysis. In addition a to DNA extracted directly from the rhizosphere samples, an enrichment procedure was carried out on samples, whereby 0.25g of collected sample material, was incubated overnight with shaking at 200 rpm at 37°C in a 2 mL microfuge tube containing either 500 µL of Luria Bertani (LB) medium (10g/L tryptone; 5 g/L yeast extract; 10g/L NaCl), or 500 µL LB supplemented with 0.1 µM GR24. This procedure was carried out on the rhizosphere samples of

each of the plant species; *P. setaceum* (Site 2), *C. ciliaris* (Sites 1 and 2), *E. cenchroides* (Site 1), *T. polystachya* (Sites 1 and 2), and *S. hirtigluma* (Site 2). After overnight incubation, the sample material suspended in the liquid medium was transferred to a ZR BashingBead™ lysis tube for DNA extraction, using the ZR Soil Microbe DNA MiniPrep™ kit.

2.4.2 Detection of Arbuscular Mycorrhizal Fungi through PCR

To determine the presence of arbuscular mycorrhizal fungi in the capping and tailing samples, a set of primers (Lee *et al*., 2008) was utilized to amplify a small subunit (SSU) on the 18S rRNA gene conserved in arbuscular mycorrhizal fungi (*Glomeromycota*). The universal AML1 and AML2 primer set targets and amplifies a small subunit rRNA gene present in AMF that produces an amplicon of 800 base pairs. The optimal PCR reagents consisted of 10 µL of 5x GoTaq® PCR buffer, 1.5 µL 10 mM dNTPs (Fermentas), 1.5 µL 10 mM forward and reverse primers (Table 2.1), 0.1 µL 5U/µL GoTaq® polymerase (Promega) and 15-35 ng of DNA in a total reaction volume of 50 µL. The optimal PCR cycling conditions are presented in Table 2.2. Each PCR reaction was accompanied with a positive control of a previously isolated 18s rRNA *Glomeromycota* DNA amplicon (isolated from garden soil and confirmed by sequencing) and a non-template control where the DNA was replaced with ddH_2O. A Bio-Rad T100™ Thermal Cycler was used to complete all PCR reactions.

Table 2.1: Primer sequences for amplification of 18S conserved regions present in AMF (Lee *et al*., 2008).

Primer name	Sequence	Product Size
AML1	5'- ATCAACTTTCGATGGTAGGATAGA -3'	800 bp
AML2	5'- GAACCCAAACAGTTTGGTTTC -3'	

Table 2.2: PCR cycling conditions for the AML1 and AML2 primer set.

PCR step	Cycling conditions		No. of cycles
	Temperature	Time	
Initial denaturation	95°C	2 min	1
Denaturation	95°C	1 min	35
Annealing (Tm)	58°C	1 min	
Extension	72°C	1 min	
Final elongation	72°C	10 min	1

2.4.3 Detection of Rhizosphere Bacteria through PCR

To determine the presence and diversity of rhizosphere bacteria a 16S primer set was utilized (Weisburg *et al*., 1991; Felske *et al*., 1997).). This primer set (Table 2.3) targets the 16S SSU rRNA gene of a phylogenetically and taxonomically wide range of bacteria. Optimal PCR reagents consisted of 10 µL 5x GoTaq® PCR buffer, 1.5 µL

10 mM dNTPs (Fermentas), 1.5 µL 10 mM forward and reverse primers (Table 2.3), 2 µL of DMSO, 1 µL 5U/ µL GoTaq® polymerase (Promega) and 15-45 ng DNA. The optimal PCR cycling conditions are presented in Table 2.4. Each PCR reaction was accompanied with a positive control of a 16S rRNA *Staphylococcus warneri* DNA amplicon (isolated from contaminated LB media and confirmed by sequencing) and non-template control of ddH_2O. A Bio-Rad T100™ Thermal Cycler was used to complete all PCR reactions.

Table 2.3: Primer sequences for the generation of 16S rRNA subunit amplicons (Felske *et al*., 1997).

Primer name	Sequence	Product Size
8F	5'- CACGGATCCAGACTTTGATYMTGGCTCAG -3'	1500 bp
1512R	5'- GTGAAGCTTACGGYTAGCTTGTTACGA CTT -3'	

Table 2.4: PCR cycling conditions to generate the 16S rRNA amplicon.

PCR step	Cycling conditions		No. of cycles
	Temperature	Time	
Initial denaturation	95°C	2 min	1
Denaturation	95°C	30 s	35
Annealing (Tm)	56°C	30 s	
Extension	72°C	1.5 min	
Final elongation	72°C	7 min	1

2.4.4 Microbial Cultures

Spread plates were also implemented to observe microbial diversity present in the collected rhizosphere tailing samples (Site 1). These tailing samples consisted of rhizosphere samples of *E. cenchroides*, *T. polystachya*, *C. ciliaris* and an open tailing area sample. This was achieved by weighing out 0.3 g of rhizosphere tailing material in a 2 mL microfuge tube and adding 1 mL of sterile dH_2O in a laminar flow cabinet. This was then vigorously vortexed for 30s. A 100 µL aliquot of this was then spread onto LB-agar (solidified with 15 g/L bacteriological agar) plates in a fume hood. A dilution series of 100x, 1000x, 10 000x and 100 000x was implemented and 100 µL of these dilutions were then spread out onto LB-agar plates. Plates were incubated at 28°C for 5 days to simulate *in situ* conditions, where after the plates were inspected to observe the establishment of microbial growth. Different microbial cultures were identified based on unique morphology characteristics, such as colour of cultures and shape of bacterial colonies. These colonies were streaked out using a metal inoculating loop on fresh LB-agar plates in a fume hood to obtain pure cultures. The plates were incubated for 5 d at 28°C. Individual colonies from the pure cultures were selected and an inoculation loop used to inoculate 1 mL of liquid LB media which was then shaken overnight at 37°C. For sample identification, PCRs were then carried out on these samples using 1 µL of

the overnight culture with the optimal PCR reagents and cycling conditions for the 8F and 1512R primer set (Section 2.4.3).

2.4.5 Visualization of DNA fragments using Gel Electrophoresis

Between 10-15 µL of each of the generated PCR amplifications were visualized through gel electrophoresis. This was achieved by the use of an agarose gel (1.5% [m/v] agarose, 0.5X TBE Buffer [5.4 g/L Tris base, 2.75 g/L boric acid, 0.465 g/L EDTA pH 8.0) stained with 2.5 µL/50 mL Pronosafe (Laboratorios Conda, Spain). The gel was placed in an electrophoresis tank and submerged in 0.5X TBE buffer, DNA fragments were separated within the gel for 1 h at 100 V. The fragments were then visualized under ultraviolet (UV) light using a G: Box F3 GeneSys gel documentation system. Amplicon sizes were confirmed by comparison against a λ *Pst*I molecular marker, which was generated by the digestion of phage λ genomic DNA with the *Pst*I restriction enzyme. The remaining volume of PCR product was then purified for cloning and sequencing using the Wizard® SV Gel and PCR Clean-Up System (Promega) as specified by the manufacturer.

2.4.6 Preparation of *Escherichia coli* DH5α cells

A modified protocol of Inoue *et al.* (1990) was utilised. *Escherichia coli* DH5α competent cells were inoculated in 2 mL LB liquid media, and incubated overnight 37°C with shaking at 200 rpm. An aliquot of 1 mL of the overnight culture was inoculated into 125 mL of liquid super optimal broth (SOB) media (5 g/L yeast extract powder; 20 g/L tryptone; 10 mM NaCl; 2.5 mM KCl; 20 mM $MgSO_4$ pH 7.5), and grown to an optical density (OD) of 0.3 at 600 nm. The temperature of the cells was lowered to 4°C, before the culture was centrifuged at 1500 *xg* for 10 min at 4°C. The supernatant was aspirated and pelleted cells gently resuspended in 40 mL ice-cold CCMB80 buffer (10 mM potassium acetate pH 7.0; 80 mM $CaCl_2$; 20 mM $MnCl_2$; 10 mM $MgCl_2$; 10% [v/v] glycerol; pH 6.4) and incubated on ice for 20 min. Resuspended cells were centrifuged at 1500 *xg* for 10 min at 4°C. The supernatant was removed and the pelleted cells resuspended in 10 mL of CCMB80 buffer. The resuspension was diluted with CCMB80 buffer until a mixture of 50 µL of the resuspended cells and 200 µL LB broth yielded an OD_{600nm} between 1.0-1.5. Aliquots of 50 µL resuspended cells were snap frozen in liquid nitrogen and stored at -80°C until use.

2.4.7 Cloning of Amplicons

The purified amplicons generated through the use of the primer sets (AML1 & 2, and 8F & 1512R) with PCR of the DNA extracted from rhizosphere samples as well as amplicons generated from the pure microbial cultures of the tailing streak plates were ligated into pGEM®-T Easy vectors (Figure 2.1), according to the manufacturer's (Promega) instructions. The ligation mixtures were transformed into *E. coli* DH5α heat-shock competent cells (Section 2.4.6). These were then plated out on LB agar containing 40 µg/mL X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 0.1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) and 100 µg/mL ampicillin and incubated overnight at 37°C.

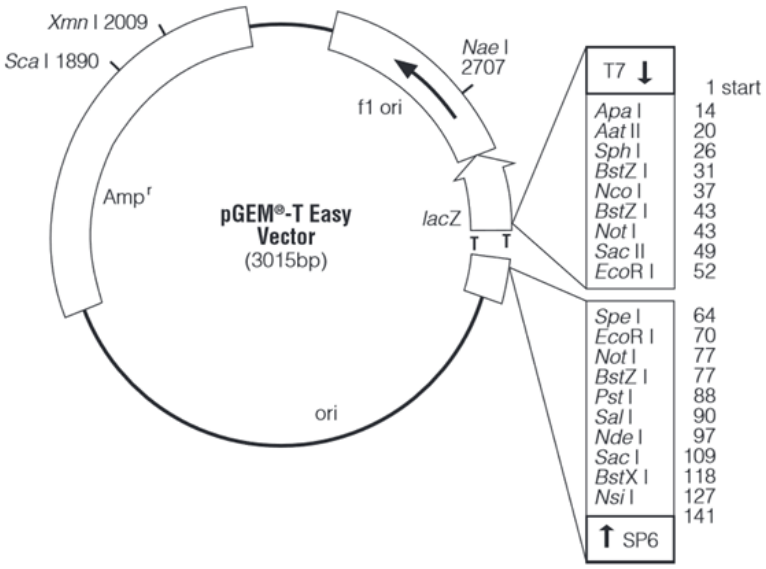

Figure 2.1: pGEM®-T Easy vector map (Promega).

2.4.7.1 Plasmid DNA Isolation

Alkaline lysis was carried out on the inoculated samples to isolate plasmid DNA. Overnight bacterial cultures were centrifuged at 16 000 *xg* for 2 min. The supernatant was aspirated and the pellet was re-suspended in ice-cold 200 µL Solution I buffer (50 mM Tris-HCl pH 8.0; 10 mM EDTA; 0.1 g/L RNAse A) by vortexing. This was followed by the addition of 200 µL Solution II buffer (200 mM NaOH; 1% [m/v] SDS) and gently mixed by inversion. Two-hundred µL ice-cold Solution III buffer (3 M potassium acetate, pH 5.5) was then added, and the tube contents thoroughly but gently mixed by inversion. The mixture was left on ice for 5 min and centrifuged at 16 000 *xg* for 10 min. The supernatant was transferred to a fresh 1.5 mL microfuge tube containing 450 µL ice-cold isopropanol. This was left on ice for 5 min to precipitate plasmid DNA, which was collected through centrifugation at 16 000 *xg* for 10 min. The supernatant was discarded and the pellet was washed with 1 mL 70% (v/v) ice-cold ethanol. The ethanol was then carefully discarded to prevent the disruption of the pellet. The tube was then left in a laminar flow-hood for 30 min to air dry the pellet. Isolated DNA was re-suspended in 50 µL ddH_2O and concentrations were measured using a Thermo Scientific Nanodrop Lite spectrophotometer.

2.4.7.2 Restriction Digestion

A total of 20 white colonies were selected from each of the transformations for restriction analysis. These transformants contained the amplicons generated from the DNA extracted from the rhizosphere samples via PCR using the AML 1 and AML 2 primers, and the 8F and 1512F primers. These 20 colonies were spotted onto new LB agar media plates containing 100 µg/mL ampicillin and grown overnight at 37°C. These colonies were then inoculated into separate tubes, each containing 2 mL liquid LB media with 100 µg/mL ampicillin, and shaken at 200 rpm overnight at 37°C. Plasmid DNA was isolated as described in Section 2.4.7.1. The isolated DNA was then treated with a variety of restriction endonucleases to develop a restriction profile of the isolated clones.

Digests were completed according to the manufacturer's instructions (New England Biolabs® Inc.) using the following enzymes; *Eco*RI, *Hin*fI, *Rsa*I, *Alu*I, and *Nci*I. Digestions were conducted by incubating 1 µg DNA with 1U of restriction enzymes, in the appropriate restriction enzyme buffer (1x), overnight at 37°C, in a total reaction volume of 20 µL. A 10 µL aliquot of each digest was then analysed by gel electrophoresis, as described in Section 2.4.6. Plasmid DNA samples that showed differing restriction patterns were selected from their respective spot plates for sequencing of the plasmid insert.

2.4.8 Sequencing

Plasmid DNA from these cultures, along with that from similar cultures from the pure cultures identified from tailing samples (Section 2.4.4**)** was then purified using the Wizard® *Plus* SV miniprep DNA purification system (Promega).The presence of the insert was confirmed through PCR using the SP6 and T7 primer set. The optimal PCR reagents used were 10 µL 5x GoTaq® PCR buffer, 1.5 µL 10 mM dNTPs (Fermentas), 1.5 µL 10 mM of SP6 and T7 (Table 3.1), 0.1 µL 5U/µL GoTaq® polymerase (Promega) and 1 µL sample inoculant and filled up to a total volume of 50 µL with ddH_2O. The optimal PCR conditions are shown in Table 3.2. A Bio-Rad T100™ Thermal Cycler was used for all PCR reactions. A 10 µL aliquot of the PCR product was analysed by gel electrophoresis, as described in Section 2.4.5 to confirm the presence of the insert. Samples were then sent for bidirectional sequencing at the Central Analytical Facility (CAF), Stellenbosch University, from the SP6 and T7 primer sites present on the vector. Plasmid vector sequences were edited using CLC Genomics Workbench (Version 8.5). The edited sequences were subjected to the Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI) to identify the organism from which the amplicon was derived to at least the genus level (Altschul *et al.*, 1990).

Table 3.1: Primer sequences of SP6 and T7 primer set

Primer name	Sequence	Product Size
SP6	5'- AATTAGGTGACACTATAG -3'	800 bp 18S and 1500bp 16S amplicons
T7	5'- AATACGACTCACTATAGG -3'	

Table 3.2: PCR conditions for SP6 and T7 primer sets.

PCR step	Cycling conditions		No. of cycles
	Temperature	Time	
Initial denaturation	95°C	5 min	1
Denaturation	95°C	1 min	30
Annealing (Tm)	45°C	1 min	
Extension	72°C	1.5 min	
Final elongation	72°C	10 min	1

3. Results

3.1 Grass species selection

Grass species were selected based on their ability to grow in disturbed areas, being indigenous to the central Lowveld, perenniality, pioneering capacity, and ideally rhizomatous (Van Oudtshoorn, 2012). The five species (*Cenchrus ciliaris*, *Anthephora pubescens*, *Chloris gayana*, *Cynodon dactylon*, and *Panicum maximum*) already used by the mine were chosen. Additional species were chosen based on the previously mentioned parameters (*Eragrostis teff*, *Paspalum notatum*, *Melinis repens*, *Stipagrostis uniplumis*, *Tragus berteronianus*, and *Enneapogon cenchroides*), and were sourced from Diverse Ecological Solutions, whilst the grass seeds collected *in situ* consisted of *Pennisetum setaceum*, *E. cenchroides*, *C. ciliaris*, and *Stipagrostis hirtigluma* which occurred frequently at the mine site (Figure 1.6). Although *P. setaceum* is considered an invasive exotic plant (Van Oudtshoorn, 2012), it was utilised during the trials due to its frequent occurrence in the mine area.

3.2 Investigation of Grass Growth and Treatments

To determine the rate that the selection of grasses could revegetate the mine dump, a number of growth parameters for each chosen species were investigated to elucidate which species would be most beneficial for the area.

3.2.1 Germination

Germination rate was measured over 43 days. In total, four trials were conducted; with a single trial consisting of four pots filled with commercial potting soil, and four pots with waste vermiculite capping material used by Palabora Copper on the mine dumps. Germination rate is regarded as the percentage of seeds that actually germinate over a period of time (Renault *et al*., 2003). Four of the commercially available species currently not used by Palabora Copper *viz. T. berteronianus*, *P. notatum*, *S. uniplumis* and *E. cenchroides*, failed to germinate, despite repeated attempts both in- and outdoors (Appendix Figures 1, 2, 3 and 4). Consequently, seed of the most common grass species growing *in situ* at Palabora Copper were also collected, to replace non-germinating species. These included *Pennisetum setaceum*, *Aristida adscensionis*, *Stipagrostis hirtigluma*, and *Enneapogon cenchroides*. Seeds from all these latter species were able to germinate, as well as the five species (*C. dactylon*, *A. pubescens*, *P. maximum*, *C. gayana* and *C. ciliaris*) utilised by Palabora Copper (Figure 3.1a and b, Figure 3.2a and b). Results from the initial trials conducted outside revealed discrepancies in overall germination rate, regarding the substrate. Although the substrate did not impact the species which germinated, germination percentages in all responsive species (with the exception of *E. teff*) were higher when germinated on the mine capping material compared to the commercial potting soil (Figure 3.1a and b). Most grass species were able to germinate within the first 3-6 days in either substrates. To minimise the number of graphs in the body of this thesis, only the figures of the first outside and greenhouse trials are displayed, with the remaining present in the Appendix.

Across all trials *E. teff* germinated the earliest, fastest and most synchronously; with most seedlings appearing within the first two weeks. This was true for both the inside and outside trials, and both substrates. Most of the *A. pubescens* seeds were observed to germinate in the mine capping material in both the outside and inside trials; however, the germination rate was higher (up to 84%) and faster in the vermiculite capping material (Figure 3.1b) than the potting soil (to a maximum of 25%, Figure 3.1a) across all trials. Most of these seedlings appeared within the first three weeks.

More *C. ciliaris* seeds germinated in both of the outside trials and an inside trial in mine capping material, than all the other trials. *C. dactylon* seeds were also shown to germinate faster in capping material across all trials than seeds germinated in potting soil, and a steady germination rate was observed. This trend was also observed with the germination rate of *M. repens* being higher in capping material than seeds grown in potting soil. The germination rate of *C. gayana* seeds were observed to be generally consistent in both substrate materials, in both outside and inside trials, as well as germinating synchronously in all replicates. Of the sourced seeds from Diverse Ecological Solutions, *P. maximum* had the lowest overall germination rate in all trials and also germinated the least synchronously of the species examined. No differences in germination rates between substrates were observed for this species. The grass species collected *in situ* from Palabora Copper germinated at varying rates, yet the same trend of more frequent germination in capping material than potting soil was observed. *P.setaceum* germinated faster than the other collected seeds, and its germination rate was the highest of the collected seeds. *A. adscensionis*, *E. cenchroides* and *S. hirtigluma* had low germination rates across all germination trials in all substrates that were conducted inside the greenhouse. These three species also germinated the least synchronously, with some germination only observed several days after the first seeds had germinated.

Changing the location of the germination rate trials to the greenhouse did not hugely alter the trends observed in the overall germination rates of the species; with the exception of *C. ciliaris*, where overall less seeds were observed to germinate throughout the duration of the greenhouse trials. The five species utilised by the mine were also observed to generally germinate at a steady rate, whereby *A. pubescens* had the greatest overall germination rate of the five species in vermiculite waste capping material.

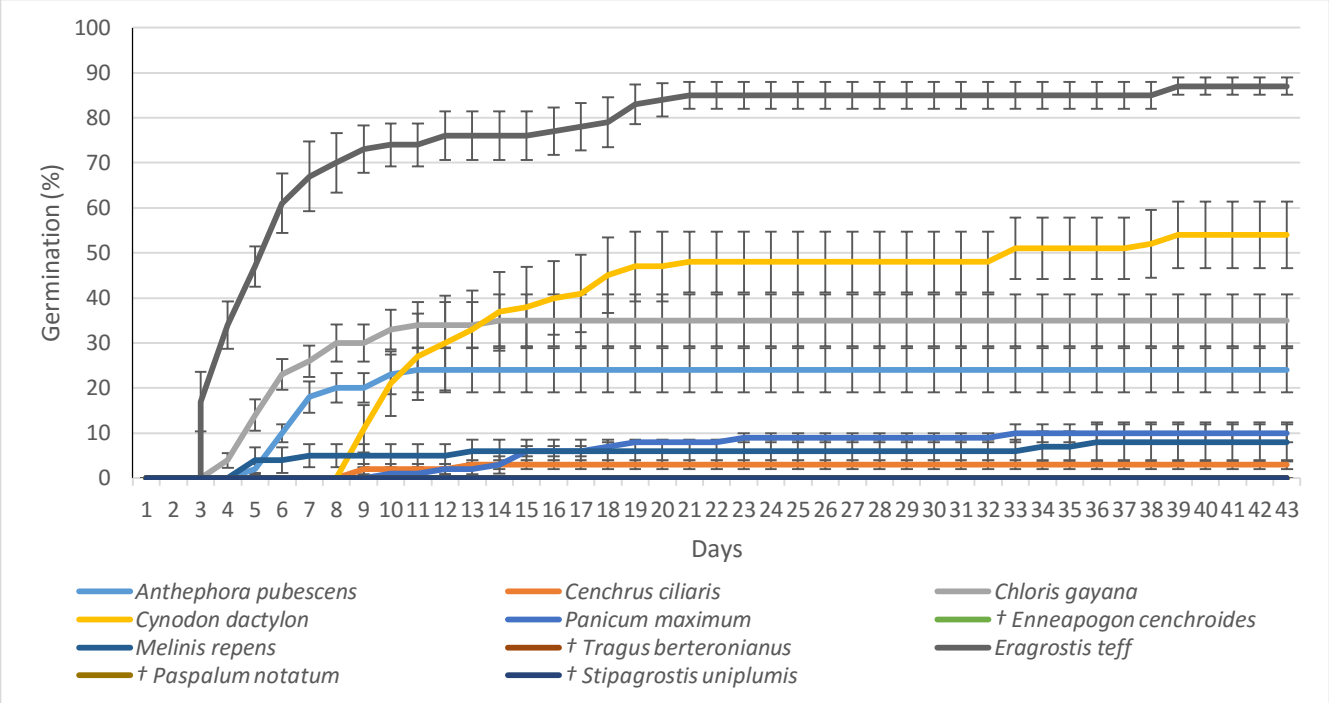

Figure 3.1a: Germination rate for seed of commercial grass species available to Palarabora Copper for use in mine dump rehabilitation. Seeds were germinated outside the IPB department, Stellenbosch University, on commercial potting soil, with daily watering during the summer. †=unresponsive species.

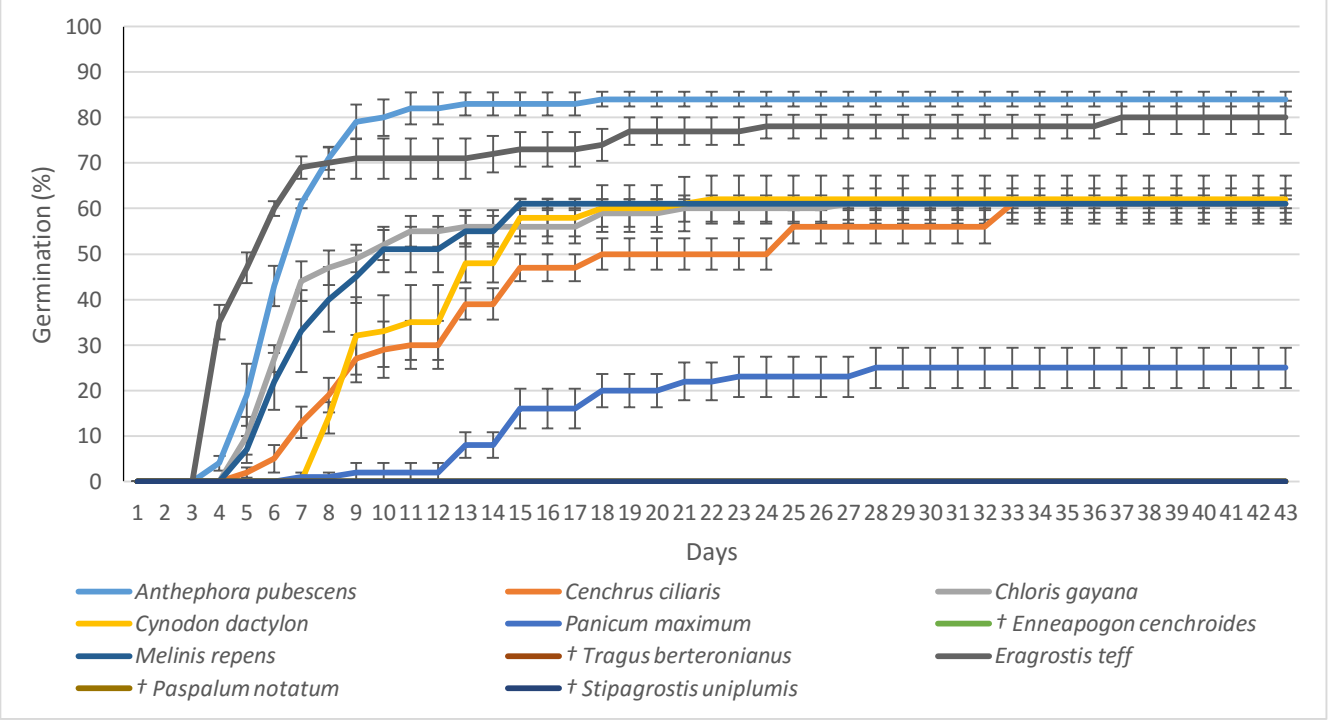

Figure 3.1b: Germination rate for seed of commercial grass species available to Palarabora Copper for use in mine dump rehabilitation. Seeds were germinated outside the IPB department, Stellenbosch University, on mine capping material used to cover the tailing dumps, with daily watering during the summer. †=unresponsive species.

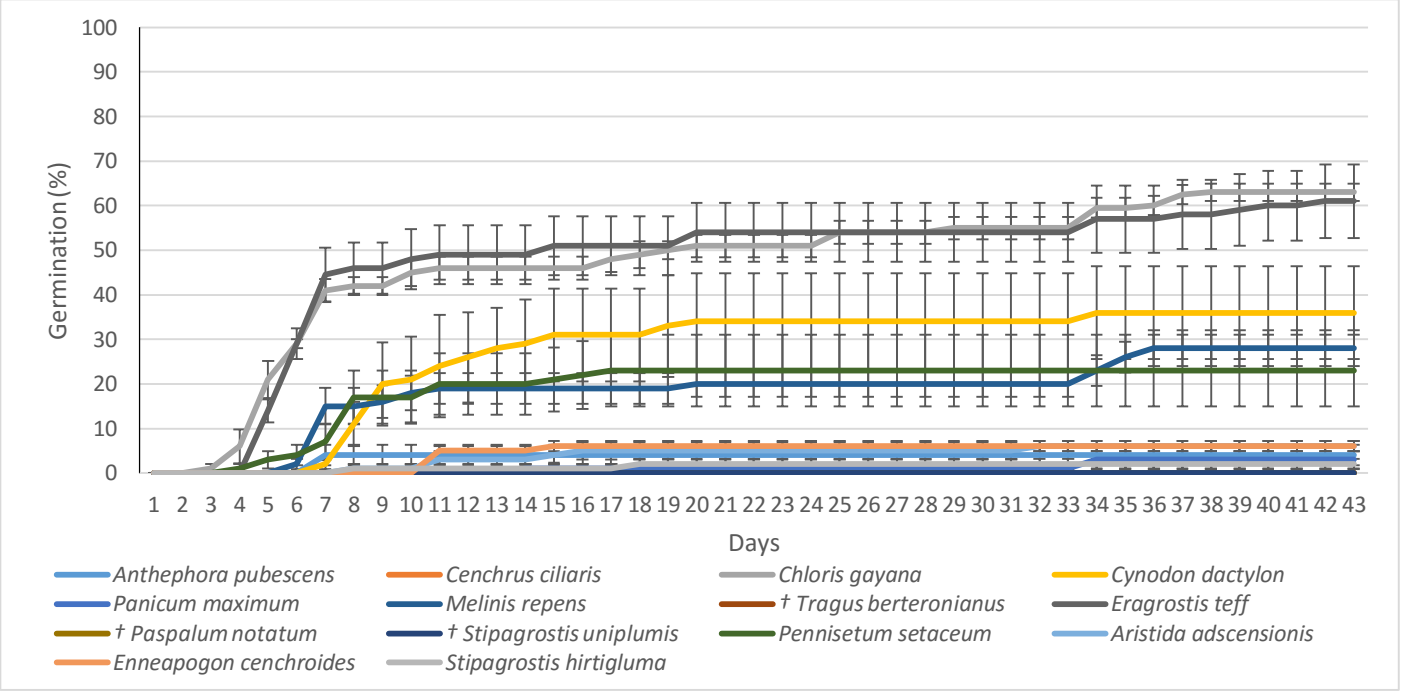

Figure 3.2a: Germination rate for seed of commercial grass species available to Palabora Copper for use in mine dump rehabilitation, as well as seed of locally dominant species occurring on site. Seeds were germinated under greenhouse conditions on commercial potting soil, with daily watering during the winter. †=unresponsive species.

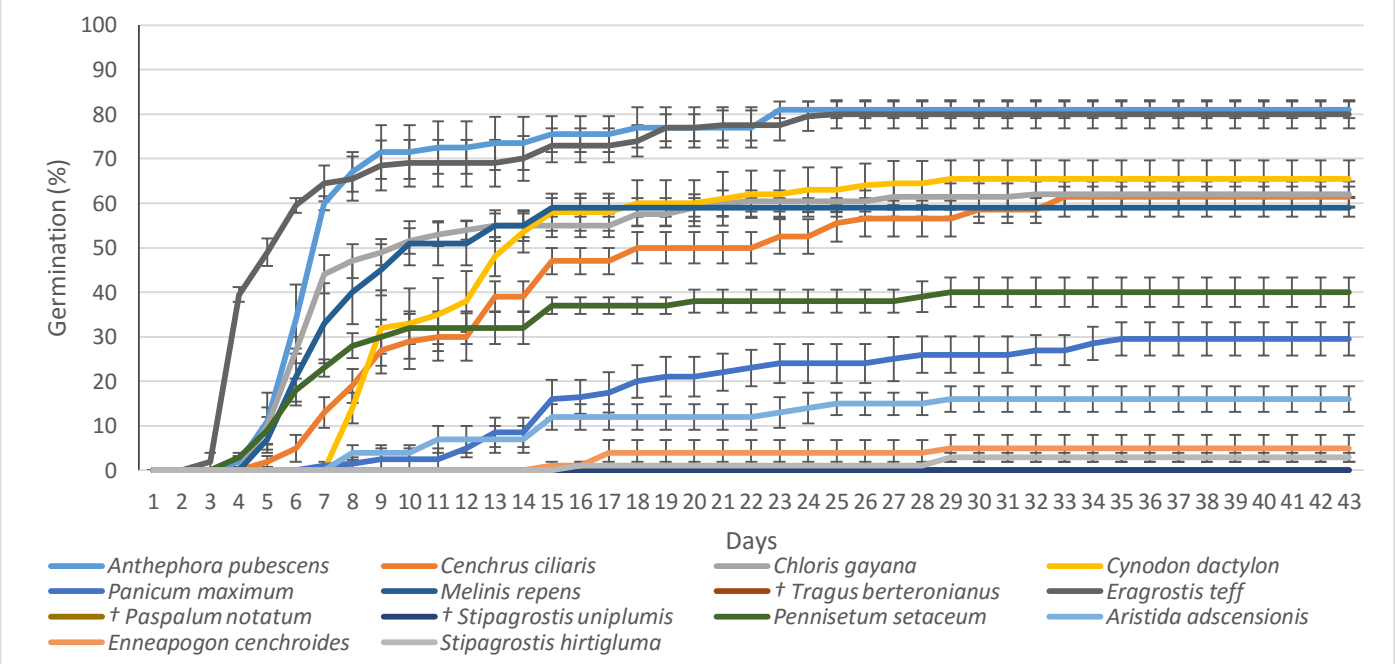

Figure 3.2b: Germination rate for seed of commercial grass species available to Palabora Copper for use in mine dump rehabilitation, as well as seed of locally dominant species occurring on site. Seeds were germinated under greenhouse conditions on mine capping material used to cover the tailing dumps, with daily watering during the winter. †=unresponsive species.

3.2.2 Growth Trials and the Influence of PGPS

For all growth trials, grass seeds were initially germinated in deep trays, filled with commercial vermiculite (Rosarium Vermiculite). At the end of the two week period, grass seedlings of similar size were selected and carefully transplanted into pots containing vermiculite capping material for the growth trials, during which the effects of a number of different plant growth promoting treatments were tested. Two major trials were conducted; the first exposed to ambient conditions and the second to greenhouse conditions, during both summer and winter. Since the commercially sourced seed of *T. berteronianus*, *P. notatum*, *S. uniplumis* and *E. cenchroides* failed to germinate, these species were not included in the growth promoter trials conducted. Seeds of those species collected during the a site visit at Palabora Copper, as described in Section 2.2, were only available for use in the later greenhouse trials. The AMF (Mycoroot™) additive was only included in the greenhouse trials as this was also not available earlier.

3.2.2.1 Outside Trials

Grass species tested in the outside trials were *C. ciliaris*, *A. pubescens*, *C. gayana*, *C. dactylon*, *P. maximum*, *E. teff*, and *M. repens*. Growth promoting treatments were applied weekly, and all pots were watered daily. After the 5 week time period, the response of seedlings to treatments was measured. Discrepancies in measured growth variables responses to growth promoting treatments within grass species, in the outside trials were observed. Seedling mortality was high during the summer especially for *P. maximum*, *E. teff*, *M. repens* and *C. dactylon*. Increased survivability of these species was observed during winter.

To minimise the number of graphs in the body of this thesis, only the figures for *C. gayana* are presented in this section. Similar figures for all of the other species tested are available in the Appendices. During the summer trial, *C. gayana*, seedlings that received lumichrome treatments showed an increase in stem length, and fresh mass relative to non-treated controls (Figure 3.3b and e). However non-treated control seedlings had longer roots (Figure 3.3c) compared to other treatments. During the winter trials, no influence was observed following any growth promoting treatments in this species, aside from the non-treated plants having a greater dry mass than all treated seedlings.

In *P. maximum*, growth responses to treatments applied during summer were predominantly insignificant. Only root length and fresh mass were positively impacted by lumichrome treatments, as compared to the non-treatment controls (Appendix Figure 5c and e). However, during the winter significant decreases in stem and root length, and fresh and dry mass were observed in GR24-treated plants relative to non-treatment controls (Appendix Figure 5h, i, k and l). Most of the *M. repens* seedlings did not survive the summer trial, and no statistical significance in the growth variables were observed. However, lumichrome treatments decreased fresh mass of *M. repens* during the winter (Appendix Figure 6k). During the summer trial, *C. dactylon* seedlings showed no statistically significant response to any treatments relative to the non-treated controls. In the winter period a decrease in leaf number was observed (Appendix Figure 7j) following lumichrome treatments relative to non-treatment controls. No significant

responses were observed during the summer trials for PGPS-treated *A. pubescens* seedlings. However, during the winter positive effects in lumichrome-treated seedlings were noted for height and stem length.

During the summer trial smoke-water increased the number of leaves in treated *C. ciliaris* seedlings (Appendix Figure 9d); however no significant effects were observed during the winter trial (Appendix Figure 9). Most of the seedlings of *E. teff* did not survive the summer trial period, with the exception being some seedlings that received either lumichrome or GR24. Lumichrome treated seedlings displayed an increase in height, stem length and number of leaves (Appendix Figure 10a, b, and d) relative to GR24-treated seedlings. During the winter period no statistical significance in seedling response to treatments were observed, as insufficient numbers of seedlings survived the winter (Appendix Figure 10).

These initial outside trials illustrated variable responses between the two conducted trials. No comparable effect in seedling responses to application of PGPS could be identified between the two trials conducted outside. Based on these results no clear trend could be identified to determine the effect of PGPS on grass seedling responses, as responses within species were too variable.

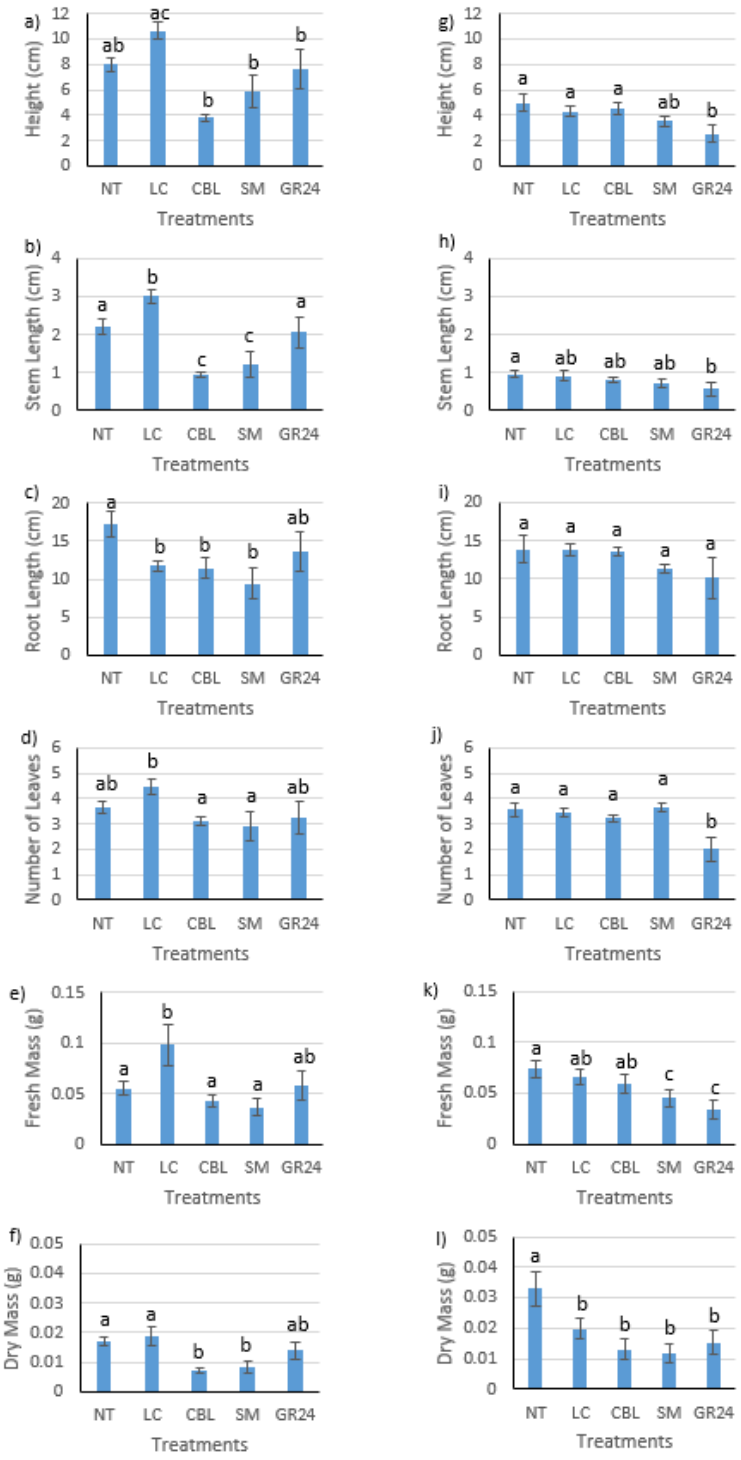

Figure 3.3: Growth of *Chloris gayana* seedlings in mine capping material during outside trials held during either the summer months (a-f) or winter months (g-l) of 2015. Parameters measured were plant height (a. g), stem length (b, h), root length (c, i), number of leaves (d, j), fresh mass (e, k) and dry mass (f, l). NT = no treatment (water only), LC = lumichrome, CBL = CropbioLife™, SM = Smoke-water, GR24 = synthetic strigolactone. Bars represent the mean of 9 replicates (n=9) ± standard error. Treatments labelled with the same letters within a graph were not significantly different from each other ($p>0.05$).

3.2.2.2 Greenhouse Trials

Grass species included in the greenhouse trials were *C. ciliaris*, *A. pubescens*, *C. gayana*, *C. dactylon*, *E. cenchroides*, and *P. setaceum*. Due to their low survivability in the previous trials, *E. teff* and *M.* repens were not included here. Smoke-water as a treatment was also eliminated due to poor performance in the outside trials, with AMF (Mycoroot™) treatments being included in its place. *Enneapogon cenchroides* and *P. setaceum* were included using seeds collected *in situ* at Palabora Copper Two additional species collected from Palabora Copper, *A. adscensionis* and *S. hirtigluma*, were initially included in these trials but failed to germinate in numbers sufficient to carry out treatment trials. The responses to the treatments in the two greenhouse trials, being winter and summer, were pooled as preliminary analyses suggested no differences between them. To determine fungal colonisation of plant roots via Trypan-Blue staining for AMF treated seedlings, all roots of the seedlings were removed. Consequently, no dry mass data was available for this treatment. *Chloris gayana* showed increases in height, stem length, root length, and number of leaves in responses to AMF treatments (Figure 3.4a, b, c, and d**)**. In *C. ciliaris*, no significant positive effect to any treatments was observed relative to non-treated controls; however, root length increased in AMF application (Figure 3.5c). No significant positive effects of treatment application were observed in *A. pubescens* seedlings, aside from an increased number of leaves following lumichrome and CropbioLife™ treatment in comparison with non-treated seedlings (Figure 3.6d). In *C. dactylon* seedlings no significant response to treatments was observed across any measured variables relative to non-treatment controls (Figure 3.7), although some treatments resulted in significantly decreased growth. GR24-treatment significantly decreased stem length in *E. cenchroides* seedlings (Figure 3.8b) relative to other treatments. *Pennisetum setaceum* seedlings responded positively to lumichrome treatment with increased dry mass, fresh mass, stem length, and number of leave. Additional positive effects were observed in CropbioLife™ treated seedlings with increased stem length and number of leaves (Figure 3.9b, d, e and f). Most, if not all the seedlings were able to survive the four week treatment trial period.

Overall, these greenhouse trials displayed variable responses to applied PGPS treatments. The AMF treatment elicited the greatest net positive responses in *C. gayana*. No statistically significant responses to applied treatments were observed in other grass seedlings relative to non-treatment controls. The CBL and GR24-treated seedlings elicited the least beneficial responses in regards to increases in measurements of growth variables.

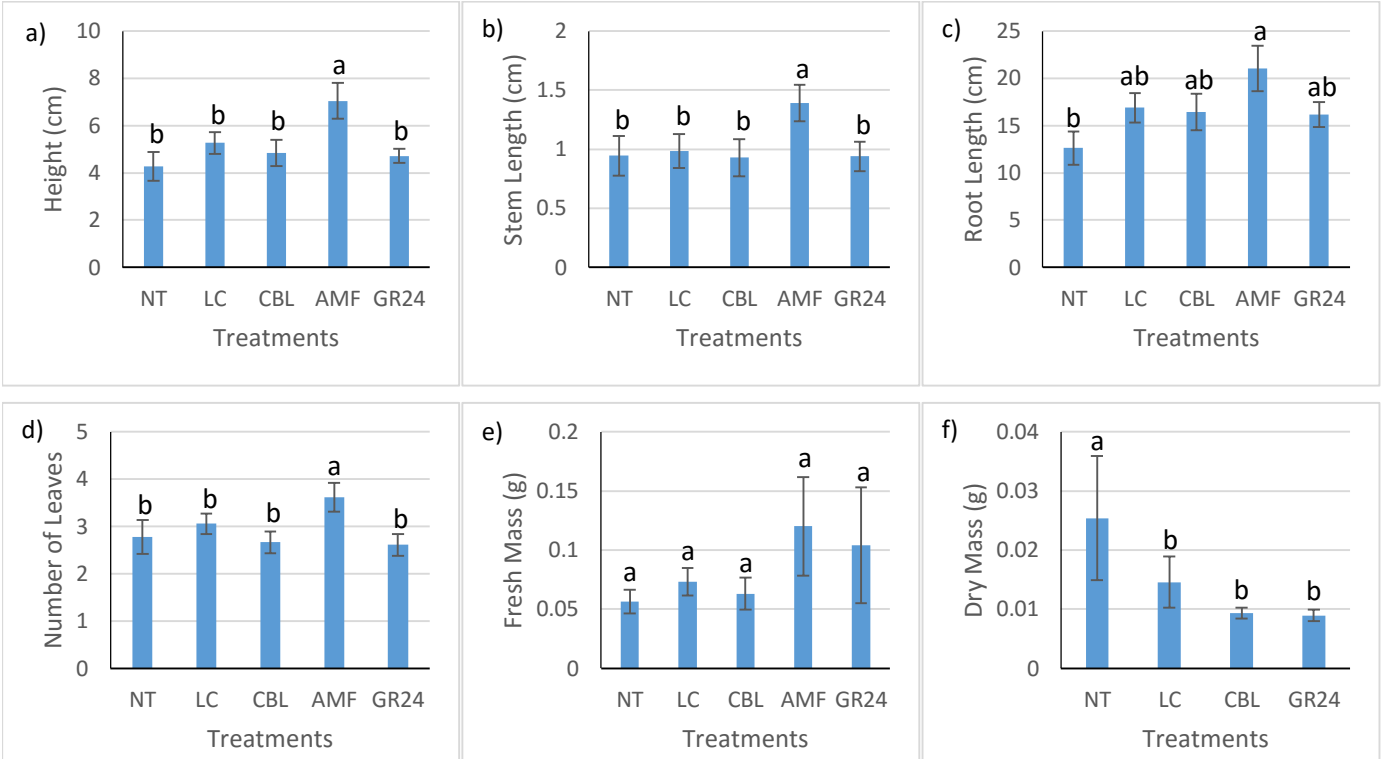

Figure 3.4: Growth of *Chloris gayana* seedlings in mine capping material during greenhouse trials of pooled treatment responses. Parameters measured were plant height (a), stem length (b), root length (c), number of leaves (d), fresh mass (e) and dry mass (f). NT=no treatment (water only), LC=lumichrome, CBL= CropbioLife™, AMF= Arbuscular mycorrhizae fungi, GR24=synthetic strigolactone. Bars represent the mean of 9 replicates (n=9) ± standard error. Treatments labelled with the same letters within a graph were not significantly different from each other ($p>0.05$).

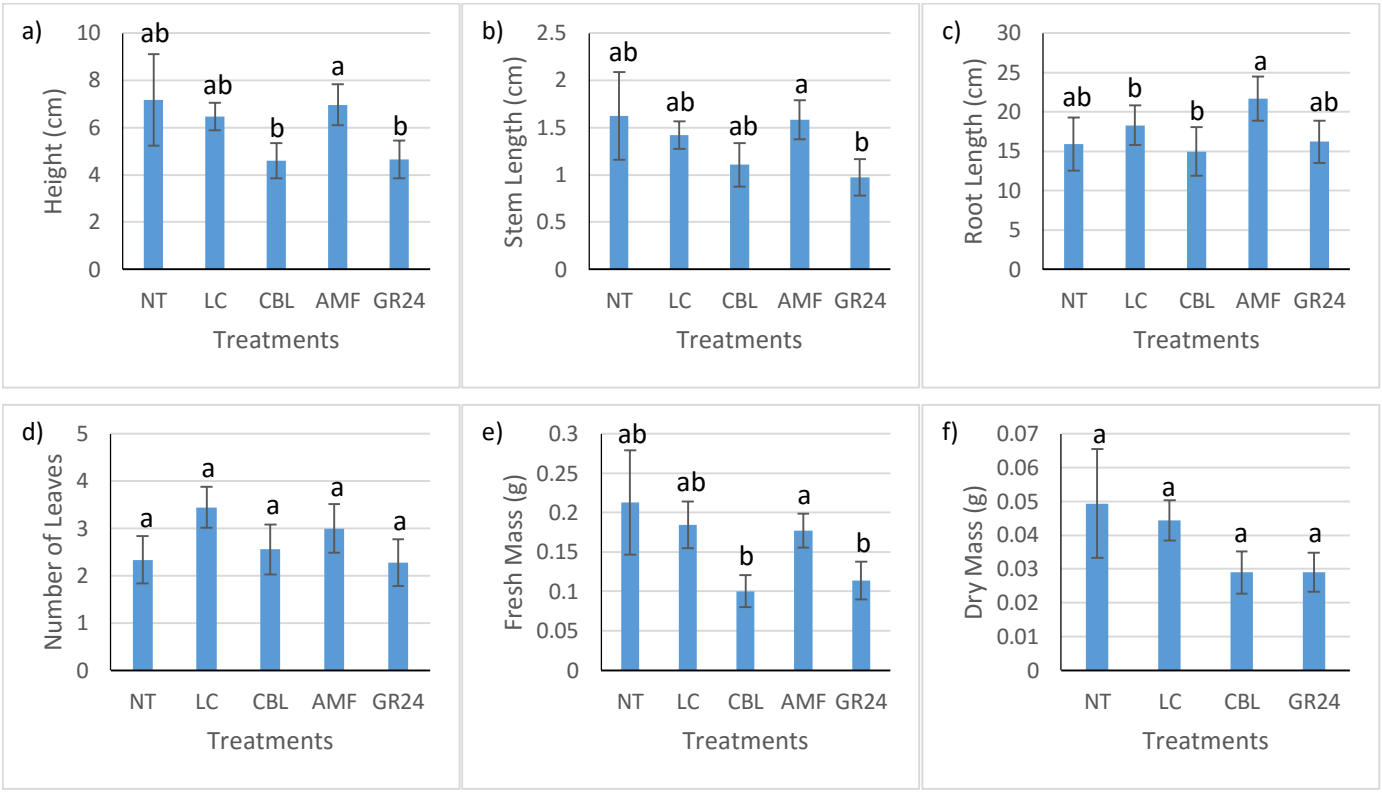

Figure 3.5: Growth of *Cenchrus ciliaris* seedlings in mine capping material during greenhouse trials of pooled treatment responses. Parameters measured were plant height (a), stem length (b), root length (c), number of leaves (d), fresh mass (e) and dry mass (f). NT=no treatment (water only), LC=lumichrome, CBL= CropbioLife™, AMF= Arbuscular mycorrhizae fungi, GR24=synthetic strigolactone. Bars represent the mean of 9 replicates (n=9) ± standard error. Treatments labelled with the same letters within a graph were not significantly different from each other (p>0.05).

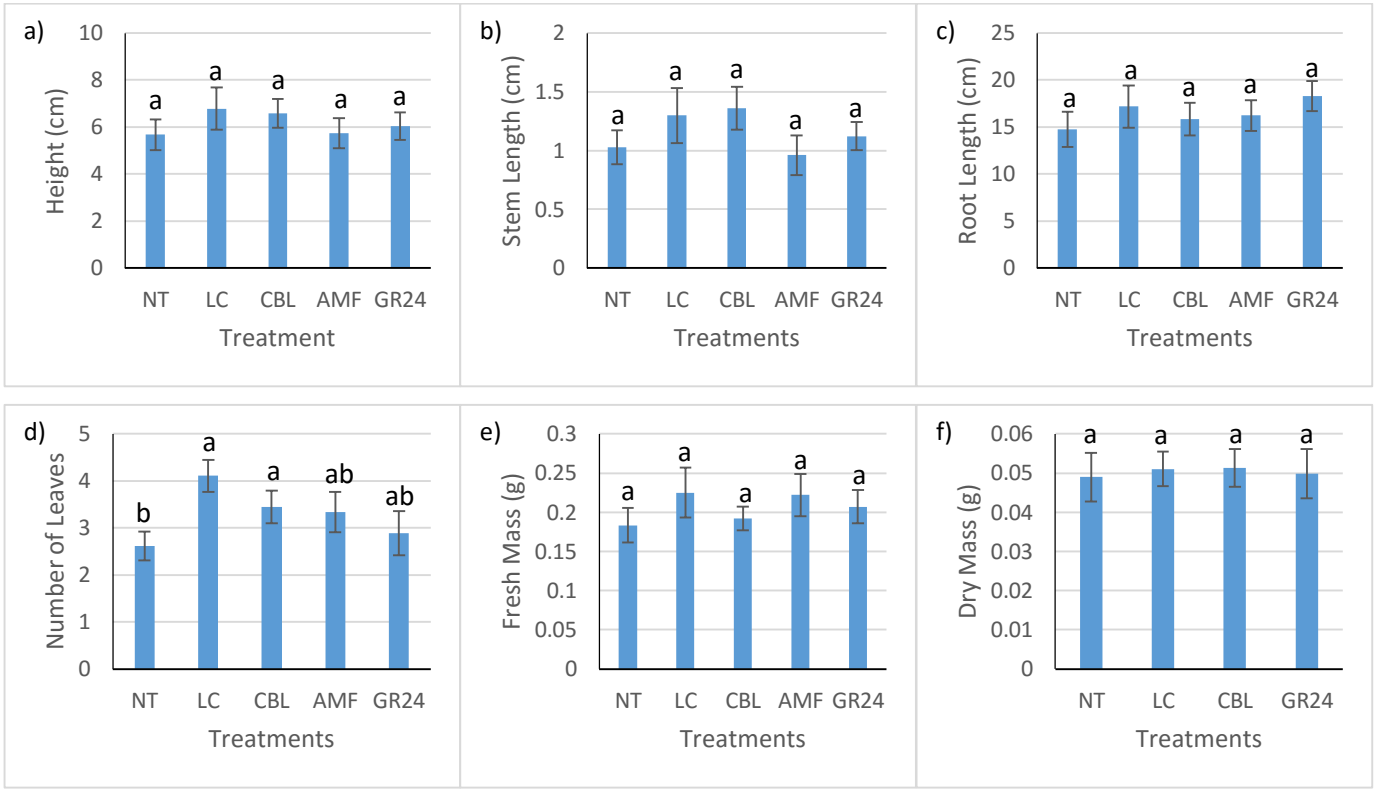

Figure 3.6: Growth of *Anthephora pubescens* seedlings in mine capping material during greenhouse trials of pooled treatment responses. Parameters measured were plant height (a), stem length (b), root length (c), number of leaves (d), fresh mass (e) and dry mass (f). NT=no treatment (water only), LC=lumichrome, CBL= CropbioLife™, AMF= Arbuscular mycorrhizae fungi, GR24=synthetic strigolactone. Bars represent the mean of 9 replicates (n=9) ± standard error. Treatments labelled with the same letters within a graph were not significantly different from each other ($p>0.05$).

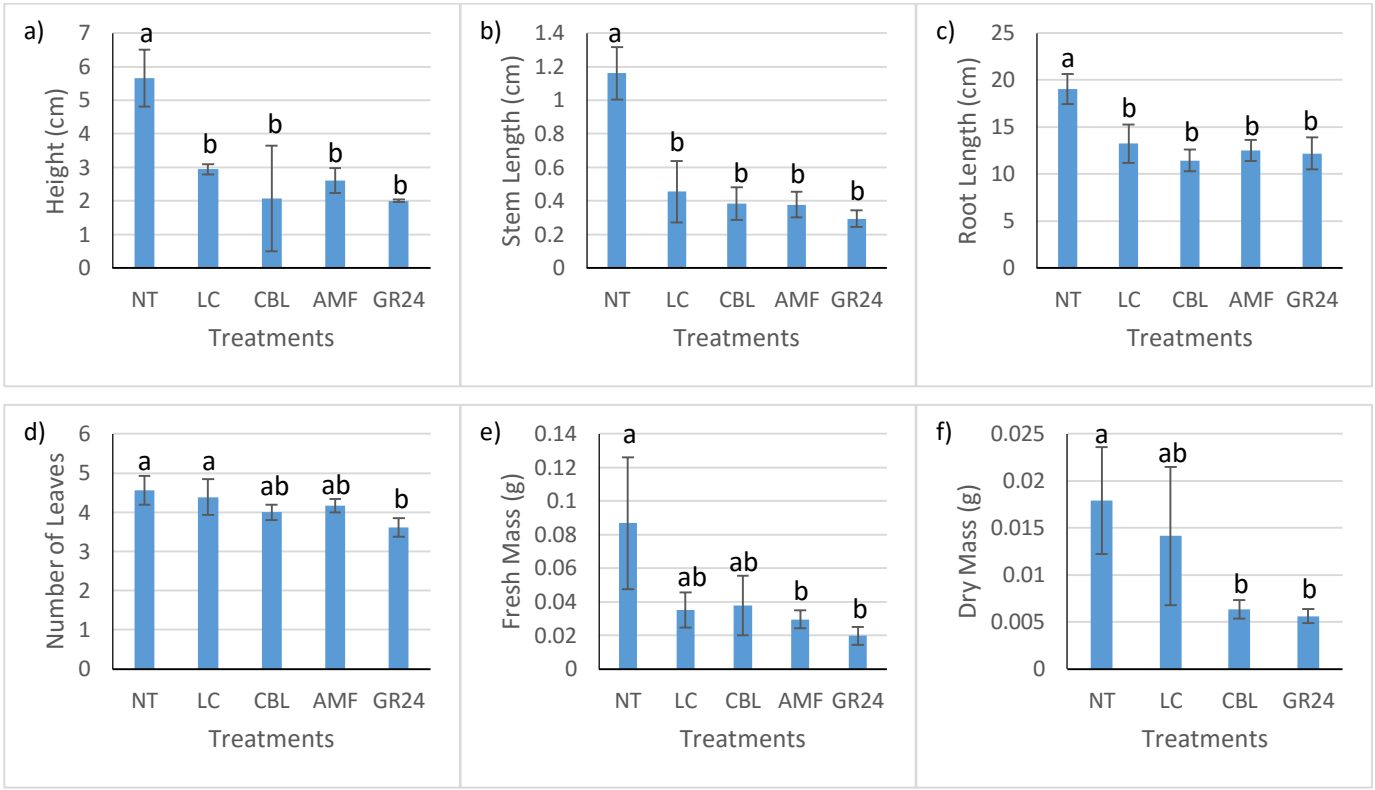

Figure 3.7: Growth of *Cynodon dactylon* seedlings in mine capping material during greenhouse trials of pooled treatment responses. Parameters measured were plant height (a), stem length (b), root length (c), number of leaves (d), fresh mass (e) and dry mass (f). NT=no treatment (water only), LC=lumichrome, CBL= CropbioLife™, AMF= Arbuscular mycorrhizae fungi, GR24=synthetic strigolactone. Bars represent the mean of 9 replicates (n=9) ± standard error. Treatments labelled with the same letters within a graph were not significantly different from each other ($p>0.05$).

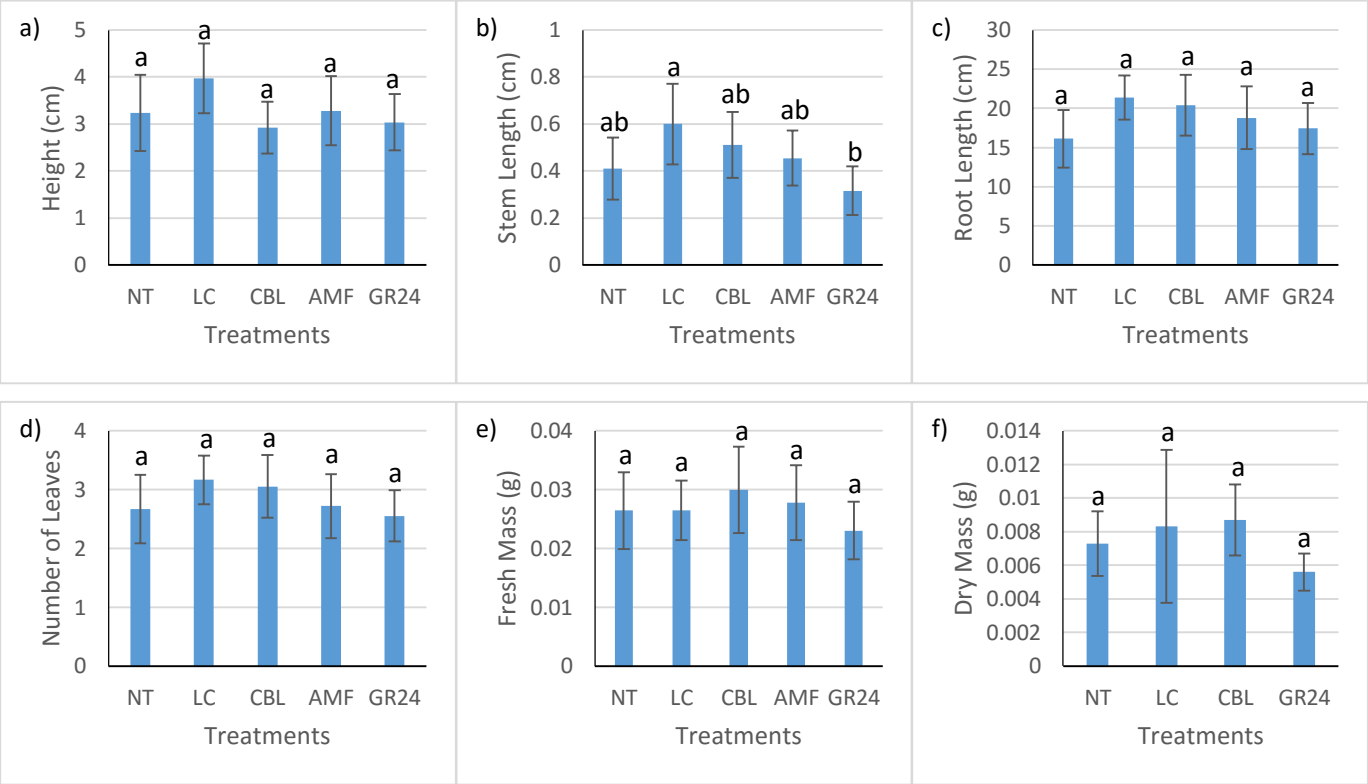

Figure 3.8: Growth of *Enneapogon cenchroides* seedlings in mine capping material during greenhouse trials of pooled treatment responses. Parameters measured were plant height (a), stem length (b), root length (c), number of leaves (d), fresh mass (e) and dry mass (f). NT=no treatment (water only), LC=lumichrome, CBL= CropbioLife™, AMF= Arbuscular mycorrhizae fungi, GR24=synthetic strigolactone. Bars represent the mean of 9 replicates (n=9) ± standard error. Treatments labelled with the same letters within a graph were not significantly different from each other ($p>0.05$).

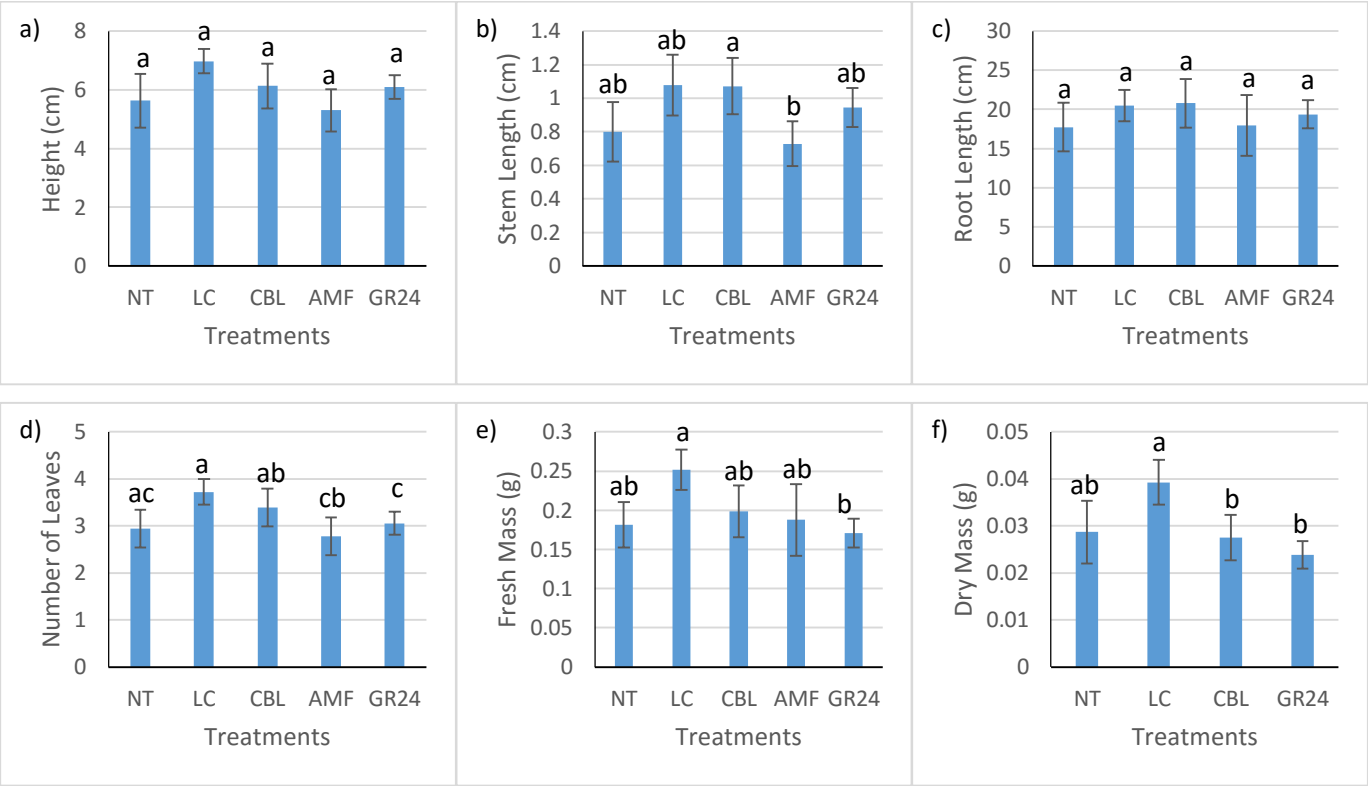

Figure 3.9: Growth of *Pennisetum setaceum* seedlings in mine capping material during greenhouse trials of pooled treatment responses. Parameters measured were plant height (a), stem length (b), root length (c), number of leaves (d), fresh mass (e) and dry mass (f). NT=no treatment (water only), LC=lumichrome, CBL= CropbioLife™, AMF= Arbuscular mycorrhizae fungi, GR24=synthetic strigolactone. Bars represent the mean of 9 replicates (n=9) ± standard error. Treatments labelled with the same letters within a graph were not significantly different from each other ($p>0.05$).

3.2.3 Trypan-Blue staining

After AMF treatments were completed, the roots of all the seedlings were collected the fresh mass was measured. Seedlings were grouped per pot (i.e. the roots of all three seedlings were pooled together) to ensure sufficient sample material was available for Trypan-Blue staining. Consequently, no dry mass data was available for this treatment. Trypan-Blue staining was carried out to determine if the selected grasses had formed symbiotic relationships with AMF (Mycoroot®). Slides of squashed root material were visualized under 40x magnification using a light microscope, to observe mycorrhizal colonisation. Colonisation was inferred based on the recognition of intercellular hyphae (McGonigle *et al.*, 1990), which appear more deeply stained than root material. No mycorrhizal colonisation was observed in *A. pubescens* in any of the treatment trials (Figure 4a). However, branching hyphal structures were observed in *C. ciliaris*, *C. gayana*, *P. setaceum*, *C. dactylon*, and *E. cenchroides* (Figure 4b-f). Roots of almost all AMF treated seedlings were observed to be colonised.

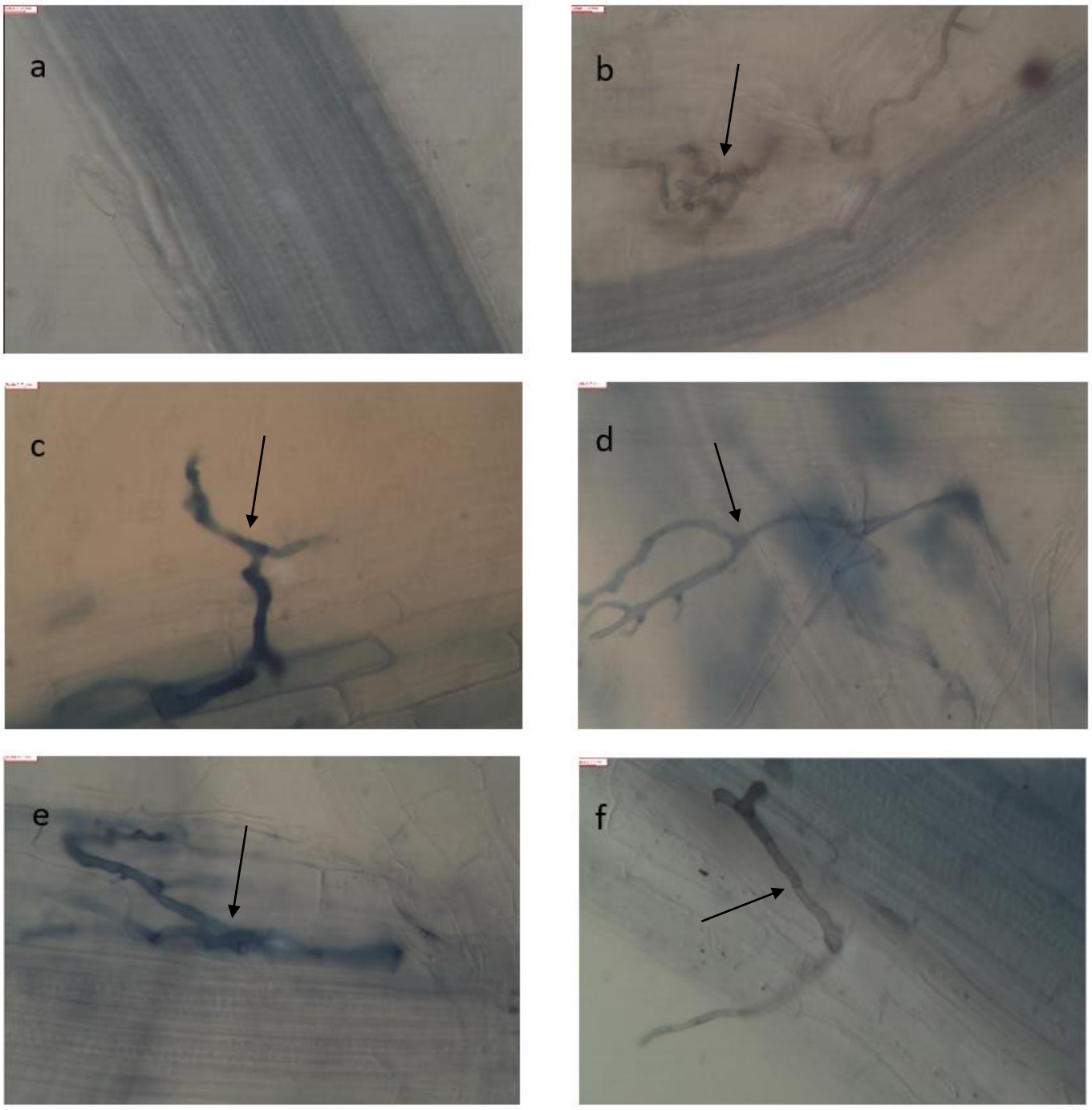

Figure 4: Trypan-Blue staining of roots of a) *Anthephora pubescens*, b) *Cenchrus ciliaris*, c) *Chloris gayana* d) *Pennisetum setaceum*, e) *Cynodon dactylon*, and f) *Enneapogon cenchroides*, following treatment with Mycoroot®. Samples were visualised under a light microscope, arrows indicating endomycota structures characteristic of mycorrhizal colonisation. Scale bar 0.70nm.

3.3 Microbial DNA Analysis

Positive AMF and bacterial controls were established from a soil sample (0.25 g) collected from a gravel parking lot at Stellenbosch University. PCR amplification from DNA extracted from this sample using AML1 and AML2 primer set for AMF detection and the 8F and 1512R primer set for bacterial detection resulted in amplicons of the expected sizes for each primer pair. These fragments were cloned into pGEM®-T Easy and sequenced. Subsequent BLAST analysis identified these inserts as uncultured *Glomeromycota* clone 46 18S ribosomal RNA gene, partial sequence (E-value 0.0, Accession Number KF939888.1) and *Staphylococcus warneri* strain G72 16S ribosomal RNA gene, partial sequence (E-value 0.0, Accession Number HQ4O7248.1) respectively. The plasmids containing these amplicons were utilised as positive controls for all subsequent PCR analyses of soil DNA extractions and colony PCRs. Initial DNA extractions, using the ZR Soil Microbe DNA MiniPrep™ kit of sample material collected at Site 1 (*C. ciliaris*, *E. cenchroides*, and *T. polystachya*) and Site 2 (*C. ciliaris*, *S. hirtigluma*, *T.polystachya*, and *P. setaceum*) at Palabora Copper yielded low amounts of DNA (11-30 ng/µL). However no PCR amplification, using the 16S rRNA (8F and 1512R) and 18S (AML1 and AML2) primer set, was possible from these samples.

3.3.1 Enrichment of Sample Material and PCR Analysis

Due to the low yield and lack of amplification from DNA extracted from the rhizosphere samples collected from some of the study sites, an enrichment procedure was implemented. This was achieved by adding either 500 µL LB medium or 500 µL of LB medium supplemented with 0.1 µM GR24 (in order to stimulate germination of AMF spores) to the rhizosphere sample and incubating overnight, extracting DNA using the ZR Soil Microbe DNA MiniPrep™ kit. This again provided relatively low DNA concentrations (15-40 ng/µL). Following PCR amplification using the universal bacterial (Felske *et al*., 1997) and AMF (Lee *et al*., 2008) primer sets on the extracted DNA samples, three of the samples produced amplicons. All three of these samples originated from site 2 (rock dump capped 10-12 years previously), which included two *P. setaceum* (designated as PennRD1 and PennRD5, respectively) and one *S. hirtigluma* (StipaRD5) samples. Both of the *P. setaceum* samples produced two 800 bp AMF and two 1500 bp bacterial amplicons (Figure 5.1), whilst the DNA extracted from the *S. hirtigluma* rhizosphere sample only produced a bacterial amplicon (Figure 5.2). The amplicons were purified, cloned into pGEM®-T Easy, and transformed into *E. coli* DH5α cells.

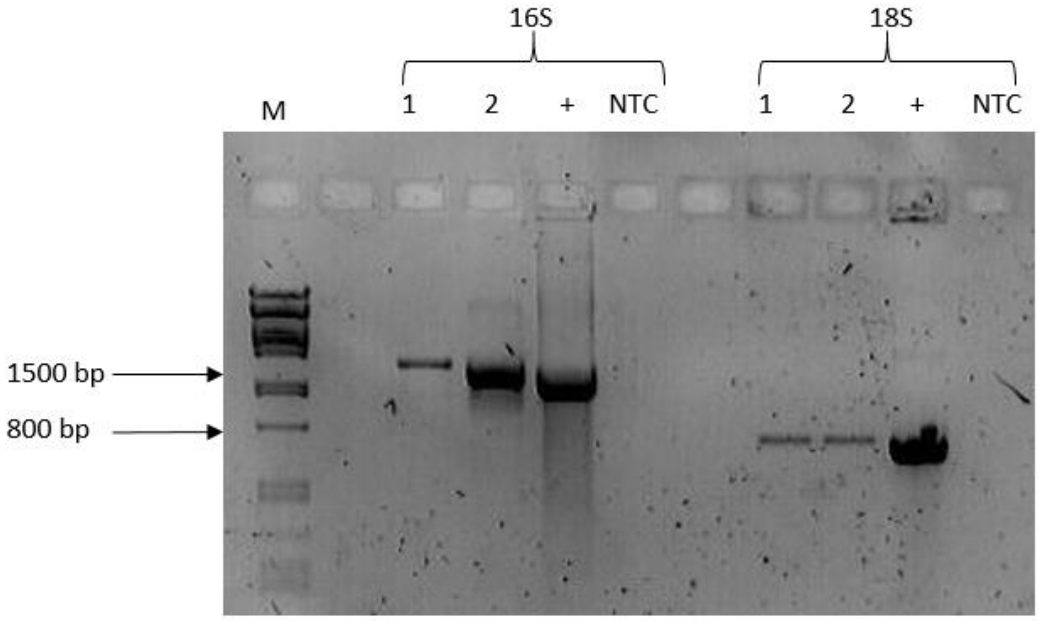

Figure 5.1: 1.5% Agarose gel showing 16S and 18S amplicons from DNA extractions of *Pennisetum setaceum* (PennRD1 and PennRD5) rhizosphere samples. 1) PennRD1 sample (LB + GR24), 2) PennRD5 (LB + GR24), M = λ *Pst*I molecular marker, + = Positive control, NTC = non-template (water) control.

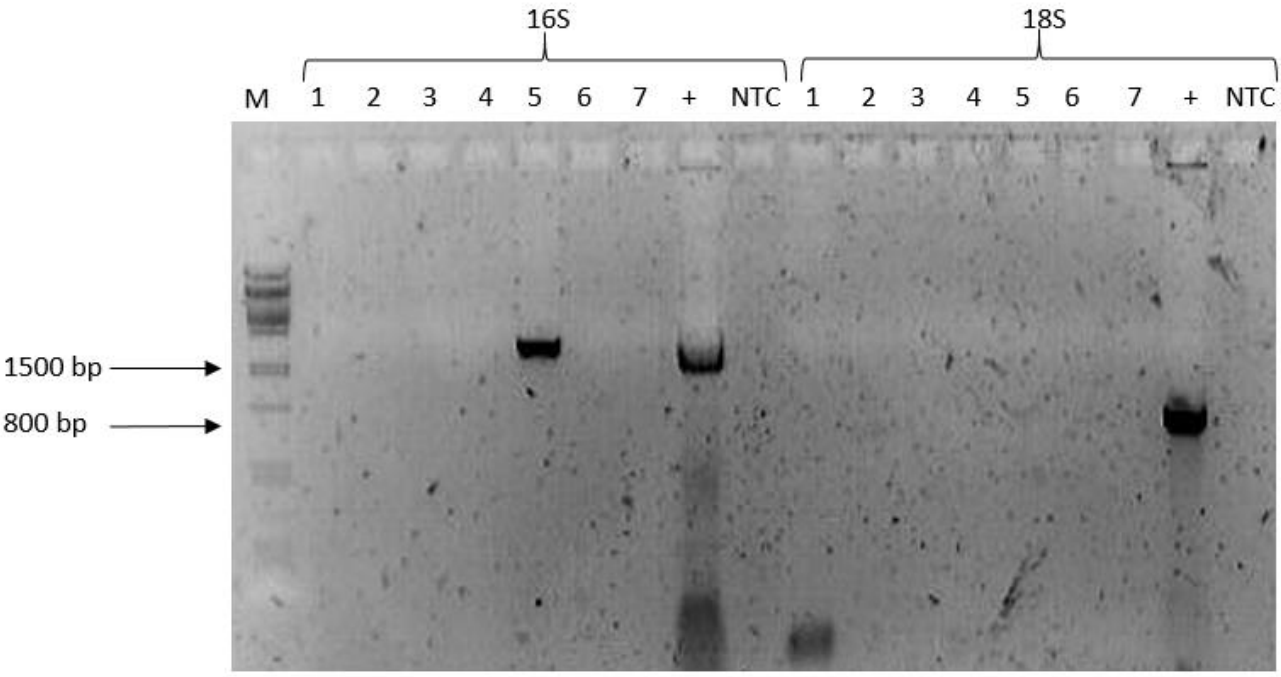

Figure 5.2: 1.5% Agarose gel showing 16S and 18S amplicons from DNA extractions of rhizosphere samples, enriched with LB media + G24. 1) *C. ciliaris* (Site 1), 2) *E. cenchroides* (Site 1), 3) *T. polystachya* (Site 1), 4) *C. ciliaris* (Site 2), 5) *S. hirtigluma* (Site 2), 6) *T. polystachya* (Site 2), 7) *P. setaceum* (Site 2), M = λ *Pst*I molecular marker, + = Positive control, NTC = non-template (water) control.

3.3.2 Restriction Analysis for Identification of Different Cloned Sequences

All of the amplicons from the rhizosphere samples were cloned into pGEM®-T Easy and transformed into *E. coli* DH5α. Following transformation, 20 recombinant colonies were selected per sample and respotted onto new LB plates. Small overnight liquid cultures were prepared for each colony and the plasmid DNA extracted.

Since the amplicons were generated from the "metagenomic" DNA samples, restriction analysis was conducted to detect polymorphism in the cloned fragments which would indicate that the amplicons had originated from different bacterial or fungal species. The same set of restriction enzymes were used as were utilized by Lee *et al*., (2008) to distinguish between variable sequences. The restriction enzyme *Eco*RI was initially used to confirm the presence of an insert in each plasmid since *Eco*RI cuts on either side of the polylinker to free the amplicon. Successive single digests were then carried out utilizing *Hinf*I, *Rsa*I, *Alu*I, and *Nci*I, to identify polymorphic inserts. Restriction analysis of the 18S rRNA amplicon from the *P. setaceum* (Site 2) sample resulted in three different restriction digest patterns (Figure 6.1), and representative colonies for all three of these were selected for sequence analysis.

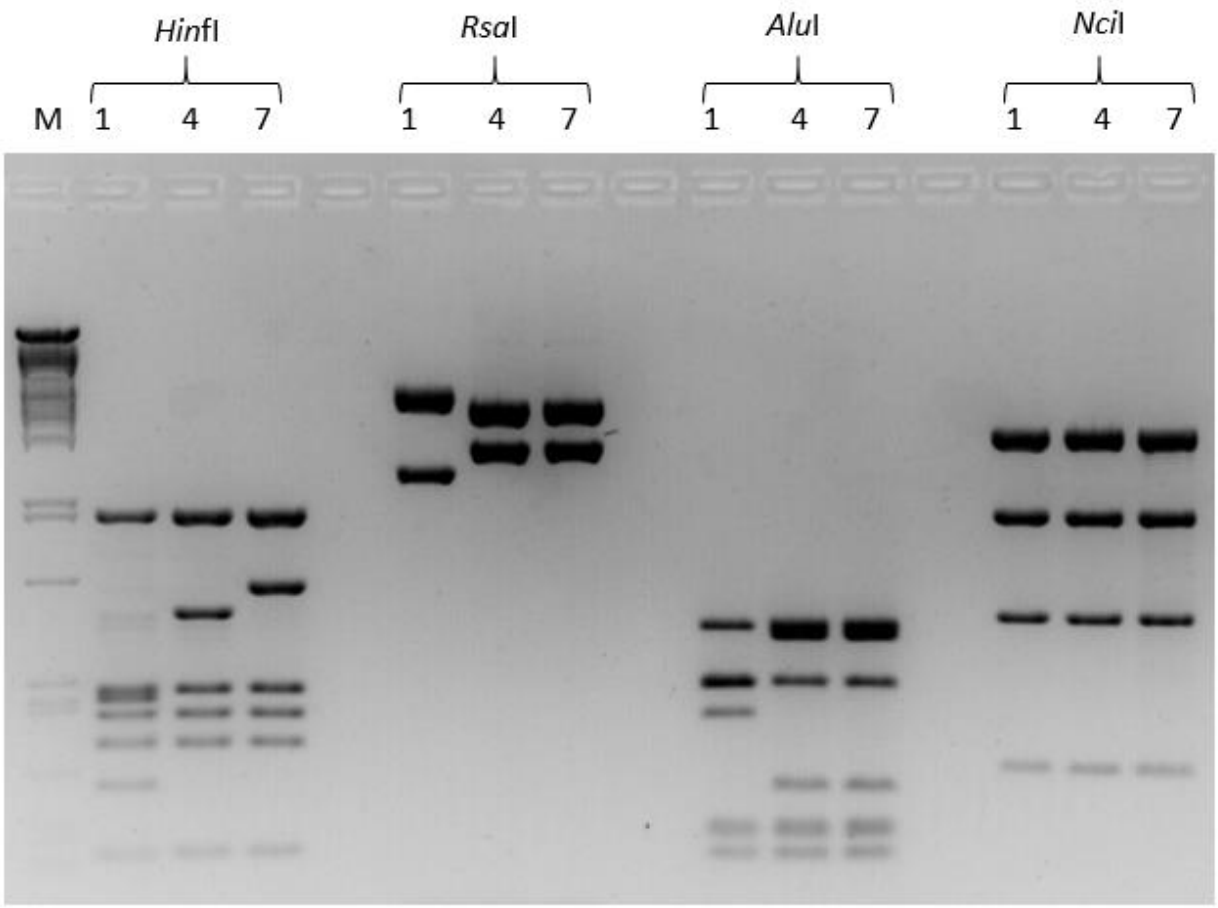

Figure 6.1: 1.5% Agarose gel showing restriction digest of 18S amplicon of purified plasmid colonies from PennRD1 (1, 4 and 7). The following restriction enzymes were used: *Hinf*I, *RsaI*, *Alu*I, and *Nci*I, M = λ *Pst*I molecular marker.

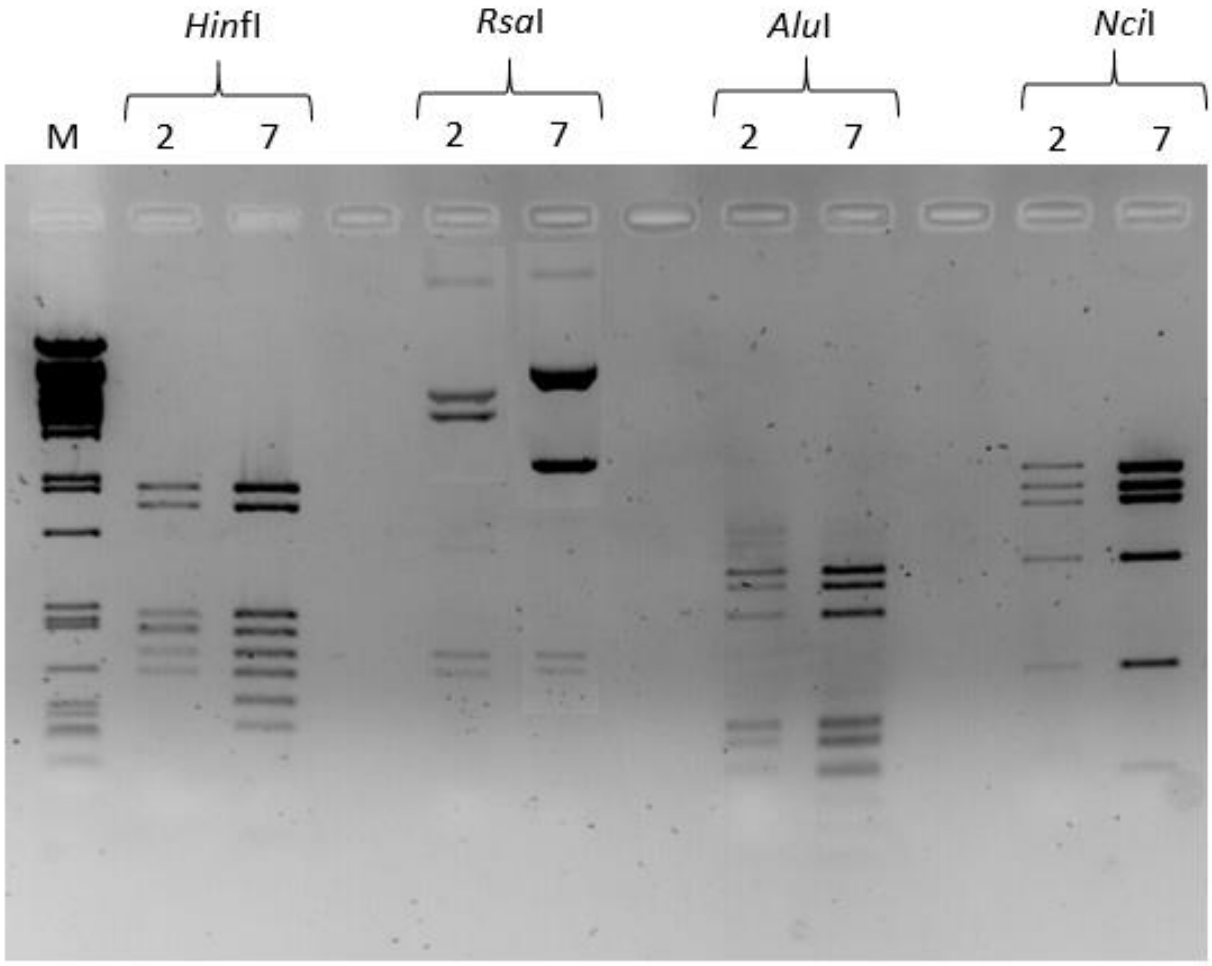

Figure 6.2: 1.5% Agarose gel visualising the restriction digest of 16S amplicon of purified plasmid colonies from PennRD1 (2 and 7).The following restriction enzymes were used: *Hin*fI, *RsaI*, *Alu*I, and *Nci*I, M = λ *Pst*I molecular marker.

This procedure was repeated for the twenty 16S bacterial (8F and 1512R) amplicons using the same set of restriction enzymes (Figure 6.2). Only two differing digestion patterns were observed amongst the 16S rRNA amplicons for the PennRD1 sample DNA (Figure 6.2). For the PennRD5 sample, three different 18S rRNA restriction patterns were identified, along with seven 16S rRNA restriction patterns.

For the StipaRD5 sample a total of nine different restriction digest patterns were identified. Plasmids from the representative colonies for each of the differing restriction patterns were sequenced from the SP6 and T7 vector specific primers. Glycerol stocks of all colonies possessing unique amplicon sequences revealed by restriction enzyme digestion patterns were prepared for long term storage at -80°C.

Sequences retrieved from CAF were assembled in CLC Genomics Workbench, and edited to remove any plasmid fragment sequences from the sequenced amplicons. All retrieved sequences were subjected to BLAST analysis, and identified to at least genus level. The 18S rRNA amplicons all had sequences of approximately 800 bp, whilst

the 16S rRNA amplicons generated from the universal bacterial (8F and 1512R) primer set yielded sequences sizes of ~1500bp. Subsequent nucleotide BLAST analyses were carried out on these sequences, and the accession numbers corresponding to the lowest E-value were recorded for each sequence Of the18S rRNA amplicons, 5 out of 6 sequences matched the *Glomus* genus (HG972964.1), whilst the sequence of a single clone matched an uncultured *Glomermycota* clone (JX296974.1) (Table 4.1).

Table 4.1: Molecular identification of 18S rRNA amplicon sequences produced by AML1 and 2 primer pair from *Pennisetum setaceum* rhizosphere samples using BLAST from NCBI.

Sample	Colony	Description	Identity %	E-value	Accession Number
PennRD1	Colony 1	Uncultured *Glomus* partial 18S rRNA gene, isolate Peraza cay, clone PE4-23T	99	0.0	HG972964.1
	Colony 4	Uncultured *Glomus* partial 18S rRNA gene, isolate Peraza cay, clone PE4-23T	99	0.0	HG972964.1
	Colony 7	Uncultured *Glomus* partial 18S rRNA gene, isolate Peraza cay, clone PE4-23T	99	0.0	HG972964.1
PenRD5	Colony 1	Uncultured *Glomus* partial 18S rRNA gene, isolate Peraza cay, clone PE4-23T	99	0.0	HG972964.1
	Colony 9	Uncultured *Glomus* partial 18S rRNA gene, isolate Peraza cay, clone PE4-23T	99	0.0	HG972964.1
	Colony 10	Uncultured *Glomeromycota* clone K1_3 18S ribosomal RNA gene, partial sequence	98	0.0	JX296974.1

The universal bacterial primer pair, 8F and 1512R, is specific for highly conserved 16S rRNA regions of bacteria, and is capable of amplifying this region from a variety of bacterial taxa (Weisburg *et al*., 1991). The amplicon sequences generated by the bacterial primers (8F and 1512R); predominantly matched the genus *Bacillus* in the enriched *P. setaceum* and *S. hirtigluma* samples. *Lysinibacillus* (KC160856.1) was dominant in the PenRD5, whilst two uncultured soil bacterium clones (HM131955.1) were also detected. Two additional uncultured bacterial clones (FM873623.1 and FJ479270.1) were identified in the StipaRD5 sample (Table 4.2).

Table 4.2*:* Molecular identification of 16S rRNA amplicon sequences produced by 8F and 1512R primer pair from *Pennisetum setaceum* and *Stipagrostis hirtigluma* rhizosphere samples using BLAST from NCBI.

Sample	Colony	Description	Identity %	E-value	Accession Number
PennRD1	Colony 2	*Bacillus* sp. CSIRPC25 16S ribosomal RNA gene, partial sequence	96	0.0	JQ580957.1
	Colony 7	*Bacillus* sp. CanR-82 16S ribosomal RNA gene, partial sequence	99	0.0	KT580672.1
PennRD5	Colony 4	*Lysinibacillus* sp. SS1.22 16S ribosomal RNA gene, partial sequence	99	0.0	KC160856.1
	Colony 5	Uncultured soil bacterium clone D2B154 16S ribosomal RNA gene, partial sequence	99	0.0	HM131955.1
	Colony 6	*Bacillus* sp. B247Ydz-hn 16S ribosomal RNA gene, partial sequence	99	0.0	EU070356.1
	Colony 7	*Lysinibacillus* sp. SS1.22 16S ribosomal RNA gene, partial sequence	99	0.0	KC160856.1
	Colony 8	Uncultured soil bacterium clone D2B154 16S ribosomal RNA gene, partial sequence	99	0.0	HM131955.1
	Colony 18	*Lysinibacillus* sp. SS1.22 16S ribosomal RNA gene, partial sequence	99	0.0	KC160856.1
	Colony 19	*Lysinibacillus* sp. SS1.22 16S ribosomal RNA gene, partial sequence	99	0.0	KC160856.1
StipaRD5	Colony 1	*Bacillus anthracis* strain A1144, complete genome	99	0.0	CP010852.1
	Colony 2	*Bacillus cereus* strain 55-3 16S ribosomal RNA gene, partial sequence	99	0.0	KM187654.1
	Colony 4	*Burkholderia* sp. RPE67 DNA, complete genome, chromosome: 3	99	0.0	AP014578.1
	Colony 6	*Bacillus* sp. B7 16S ribosomal RNA gene, partial sequence	99	0.0	KF479571.1
	Colony 10	*Bacillus* anthracis strain A1144, complete genome	99	0.0	CP010852.1
	Colony 15	Uncultured bacterium partial 16S rRNA gene, clone MA01F08	97	0.0	FM873623.1
	Colony 20	Uncultured bacterium clone p26n15ok 16S ribosomal RNA gene, partial sequence	95	0.0	FJ479270.1

3.3.3 Molecular Identification of Bacterial Spread plates

Four sets of spread plates per rhizosphere sample (*C. ciliaris*, open tailing area, *E. cenchroides*, and *T. polystachya*) collected from Site 1 were spread out on in a dilution series on LB agar plates (Figure 7.1**)**. The optimal dilution appeared to be 1000x, as this allowed individual colonies to be identified whilst still maintaining relatively large diversity of species. Different species were visually identified based on unique morphological characteristics, and pure culture plates prepared for each. Single colonies were picked from the pure cultures and inoculated into 1 mL LB media and incubated overnight. A 1 µL aliquot from each culture was then used as the template for PCR using the 16S rRNA gene bacterial (8F and 1512R) primer set.

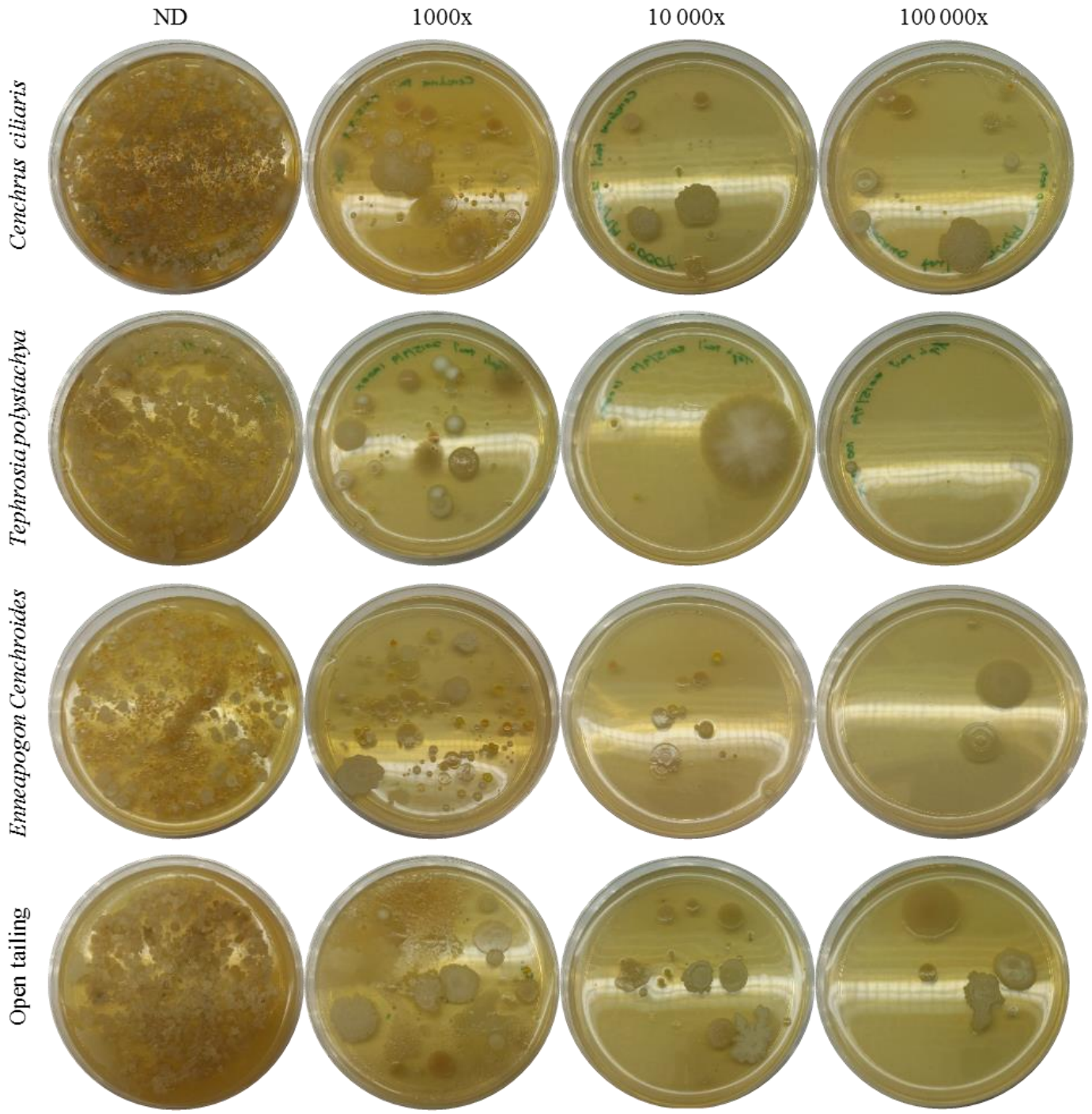

Figure 7.1: Spread plates of collected rhizosphere tailing samples grown on LB agar. ND=Non-diluted

Four unique cultures were identified from the Open Tailings 1000x dilution plate. Only three of these colonies produced amplicons after PCR (Figure 7.2). Six amplicons were similarly isolated from the *C. ciliaris* rhizosphere sample, a further 3 from the *E. cenchroides* rhizosphere sample and another 5 from the *T. polystachya* sample. All amplicons were purified and submitted for sequence analysis. These amplicons were ligated into pGEM®-T Easy vector, and transformed into competent *E. coli* DH5α cells. Recombinant colonies were identified and selected from the plates, and inoculated in 3 mL liquid LB medium and incubated at 37°C overnight. Colony PCRs using the SP6 and T7 primers were used to confirm the presence of the insert in the cultures before plasmid DNA was isolated and sequenced. The sequences that were obtained were edited and subjected to BLAST analysis. All sequences corresponded to highly conserved 16S rRNA bacterial regions. The accession number and details of the BLAST hit with the lowest E-value was recorded for each sequence (Table 5.1). The sequences were predominantly members of the *Bacillus* genera. The amplicon sequences generated from the bacterial cultures isolated from the rhizosphere of the unvegetated tailing sample matched three separate sequences; *Brevibacterium halotolerancs* (JX644589.1), uncultured *Firmicutes* bacterium (GQ249508.1), and *Fictibacillus barbaricus* (KJ575018.1). The three bacterial amplicons produced from the *E. cenchroides* rhizosphere sample were identified as *Bacillus licheniformis* (NR_074923.1), an uncultured bacterium clone (AB636955.1), and a *Bacillus* (KM873110.1) species clone. Two of the *T. polystachya* rhizosphere sample sequenced amplicons were identified to be from the genus *Bacillus* (KF956662.1, and EU798946.1), two cultures identified as *Bacillus vietnamensis* (JQ799107.1), and one as an uncultured *Actinobacterium* (JN037883.1). The PCR products produced from the *C. ciliaris* rhizosphere sample were identified as two *Cupriavidus* (LC065169.1) isolates, *Arthrobacter* (JQ793579.1), *Bacillus* (EU685816.1), *Paracoccus siganidrum* (KM015451.1) and *Roseomonas* (LN810637.2).

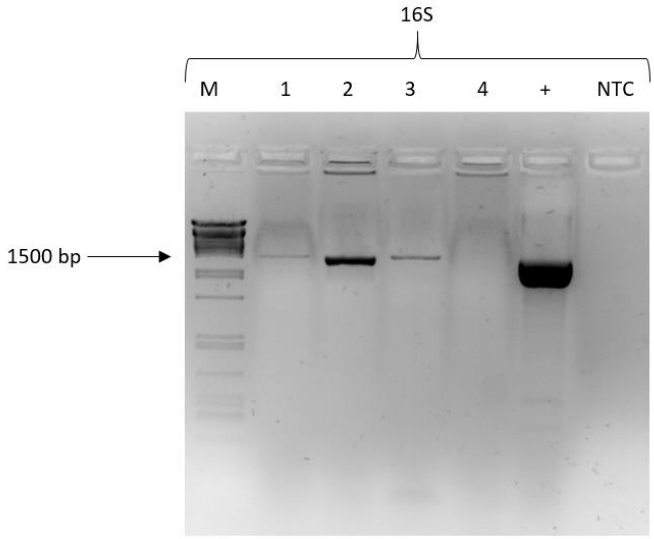

Figure 7.2: 1.5% Agarose gel showing 16S amplicons from unvegetated (open plate) tailing 1000x dilution plate colonies 1, 2, 3, 4, M = λ *Pst*I molecular marker, + = Positive control, NTC = non-template (water) control. The sizes of the generated amplicons are approximately 1500 bp.

Table 5.1: Molecular identification of rhizosphere bacteria cultures from amplicon sequences produced by the bacterial primer pair (8F and 1512R). Amplicon sequences were identified using BLAST from NCBI.

Sample	Colony Plate	Description	Identity %	E-value	Accession Number
Open tailing	Colony 1	*Brevibacterium halotolerans* strain CAS17 16S ribosomal RNA gene, complete sequence	99	0.0	JX644589.1
	Colony 2	Uncultured *Firmicutes* bacterium clone B02 16S ribosomal RNA gene, partial sequence	99	0.0	GQ249508.1
	Colony 3	*Fictibacillus barbaricus* strain NIOT-Ba-23 16S ribosomal RNA gene, partial sequence	99	0.0	KJ575018.1
***Enneapogon cenchroides* rhizosphere**	Colony 1	*Bacillus licheniformis* strain ATCC 14580 16S ribosomal RNA gene, complete sequence	99	0.0	NR_074923.1
	Colony 2	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: 12TCLN029	97	0.0	AB636955.1
	Colony 6	Uncultured *Bacillus* sp. clone ACH-14S-304 16S ribosomal RNA gene, partial sequence	99	0.0	KM873110.1
***Tephrosia polystachya* rhizosphere**	Colony 2	*Bacillus* sp. S12206 16S ribosomal RNA gene, partial sequence	99	0.0	KF956662.1
	Colony 4	*Bacillus* sp. JY01 16S ribosomal RNA gene, partial sequence	99	0.0	EU798946.1
	Colony 6	*Bacillus vietnamensis* strain KJ-W10 16S ribosomal RNA gene, partial sequence	99	0.0	JQ799107.1
	Colony 7	Uncultured *Actinobacterium* clone UHAS5.56 16S ribosomal RNA gene, partial sequence	99	0.0	JN037883.1
	Colony 8	*Bacillus vietnamensis* strain KJ-W10 16S ribosomal RNA gene, partial sequence	99	0.0	JQ799107.1
***Cenchrus ciliaris* rhizosphere**	Colony 1	*Cupriavidus* sp. NCCP-1142 gene for 16S ribosomal RNA, partial sequence	99	0.0	LC065169.1
	Colony 2	Uncultured *Arthrobacter* sp. clone SGR270 16S ribosomal RNA gene, partial sequence	99	0.0	JQ793579.1
	Colony 4	*Bacillus* sp. PK-14 16S ribosomal RNA gene, partial sequence	99	0.0	EU685816.1
	Colony 5	*Cupriavidus* sp. NCCP-1142 gene for 16S ribosomal RNA, partial sequence	99	0.0	LC065169.1
	Colony 6	*Paracoccus siganidrum* strain 3UW2 16S ribosomal RNA gene, partial sequence	99	0.0	KM015451.1
	Colony 7	*Roseomonas* sp. JC288 partial 16S rRNA gene, strain JC288	99	0.0	LN810637.2

4. Discussion

4.1 Grass Germination Trials

The primary motive of this research was to develop methods to enhance grass establishment on mine tailings, through identification of optimal grass species and possible treatments to enhance plant growth and establishment. Covering these tailings with vegetation would prevent erosion by wind, enhance water infiltration and initiate the first stages of ecosystem processes and plant succession. A secondary aim of the germination trials was to investigate the influence of soil nutrients on the germination of species when grown in nutrient poor mine vermiculite capping material versus nutrient rich commercial potting soil. Additionally, these trials would assess seed germinability of commercially-sourced and *in situ* collected seeds to determine their germination capabilities in differing substrates. Furthermore, the germination trials provided useful information on whether it was possible to germinate the seeds directly in the capping material to utilise in the PGPS trial, or if mass pre-germination of seeds were needed and select similar sized seedlings for the trials. During the germination rate trials of the various grass species, results were obtained to indicate which species might be the most useful in revegetating the tailings.

To identify the optimal grass species for revegetation purposes of mine tailings operated by Palabora Copper, the germination rates and germinability of a selection of grasses were investigated by germinating seeds in nutrient-poor mine capping material and nutrient-rich potting soil. Seeds that did not germinate over the trial period were regarded as unviable and were eliminated from subsequent PGPS treatment trials. The species that did not germinate included four grasses sourced from Diverse Ecological Solutions (*Tragus berteronianus*, *Enneapogon cenchroides*, *Stipagrostis uniplumis*, and *Paspalum notatum*). Additionally, two grass species seeds collected *in situ* from Phalaborwa (*Stipagrostis hirtigluma* and *Aristida adscensionis*) not germinate in numbers sufficient to support the amount of seedlings necessary for the PGPS trials, and were also discarded from the trials. The germination trials also revealed mostly asynchronous germination within and between species, meaning that seed for the PGPS trials could not be germinated directly in pots containing the mine capping material.

Initially, germination experiments were performed in an uncontrolled, outdoor environment, as this would best simulate those conditions the grasses would be exposed to on the mine dumps. These trials revealed inconsistent germination rates between summer and winter repeats. This can be attributed to the change in seasons, as the first trial was conducted during summer from the end of January to mid-March, whilst the second was conducted during autumn/winter from the beginning of April to mid-May. As a result, the germination rate of grass seeds of most species was delayed during the winter i.e. less germination during the winter period in both potting soil and mine capped material. Conversely, *C. ciliaris* and *P. maximum* showed improved germination rates on the mine capped material during the winter trial period. *Cenchrus ciliaris* has been shown to be a dominant species in mined areas with poor nutrient conditions (van Rensburg *et al*., 2003; Liebenberg and Rensburg, 2013) thereby indicating its capacity to grow in the mine capping material. *P. maximum* is known to grow in most soil types albeit in moist,

well-drained and fertile soil (Van Oudtshoorn, 2012). An environmental effect can be attributed to the retardation in germination rates across the winter trial period (Appendix Figure 1 and 2). It should be borne in mind that because these experiments were performed *ex situ* in Stellenbosch (Western Cape), the germination rate for the various species may be different from those that might be obtained *in situ* in the South African Lowveld. Results regarding the germination rate obtained in Stellenbosch might therefore not relate with results at Palabora Copper. These trials received daily watering, which would not be the case in the Limpopo province which receives an average of 50 mm monthly rainfall. The differences in climates between the Stellenbosch and Phalaborwa would subsequently have an impact on the growth of the grass species.

To exclude environmental influences on germination rates, a second set of trials was conducted in a greenhouse with a constant photoperiod of 16 h light and 8 h dark per day, with a relatively constant temperature (average day and night temperatures 20°C to 27°C for the first trial and 22°C to 29°C for the second trial) across the trial periods. All grass species, commercially sourced and *in situ* collected seeds, were included in these trials. Overall, germination rates inside the greenhouse corresponded with results attained during the first outside trial. However, there was a marked decrease in the germination rate of *C. ciliaris* in the inside versus the outside trials, bar the first greenhouse trial using mine capping material.

There was a marked difference between the germination rates of seeds based on the nutrient status of the germination substrate. In all trials, the germination rates was higher for grass species in the nutrient-poor mine capping material as opposed to the nutrient-rich potting soil. This effect was present in both outside and inside trials. *Chloris gayana* germination rates were mostly consistent throughout all trials on both substrates, with no significant differences observed between inside or outside trials. This species has been utilised widely in revegetation purposes on nutrient poor soils (Mentis, 1999; van Rensburg *et al*., 2003; Rensburg and Morgenthal, 2004). Aside from poor germination rates observed for the inside trials for *C. ciliaris*, most grass species performed similarly to the outside summer period trial. The favourable germination rates of the perennial grasses *A. pubescens, C. dactylon, M. repens, C. gayana,* and the annual *E. teff* on mine capping material suggest that these species would be of greatest value to Palabora Copper for revegetation purposes (Liebenberg and Rensburg, 2013; Granger and O'Connor, 2014). These grasses have been noted for their occurrence in disturbed soils (Van Oudtshoorn, 2012), which explains their generally high germination rate in mine capping material as well as their widespread use by mining companies to revegetate areas of poor soil nutrient status (van Rensburg *et al*., 2003; Rensburg and Morgenthal, 2004).

It is worth noting that despite the lack of nutrients and structural support that the mine capping material provides for plant growth, most of the tested grass species germinated at a higher and more rapid rate on this substrate than those grown in potting soil. Although the presence of readily available nutrients in the potting soil should have provided plants with increased survival and germination rates, this was not observed in regards to some grass species' ability to germinate in the nutrient poor vermiculite waste capping material. Based on the observations,

and the stark differences observed in germination rates across the different substrates, the grasses that were able to germinate the fastest and most synchronously were *A. pubescens*, *C. dactylon*, *E. teff*, *M. repens*, and *C. gayana*.

4.2 Plant Growth and Growth Enhancing Treatments

Initial treatment trials, which were conducted outside, revealed inconsistencies in terms of growth variables in response to PGPS treatments. The variables that were measured as proxies for seedling vigour and therefore survivorship, were chosen to determine if treatments would offer potential benefit to phytostabilisation by improving plant growth and survival in a disturbed area affected by heavy metal contamination. Specifically, the aim of the study was to determine if the application of selected PGPS on a number of grass species could improve plant establishment to the benefit of the revegetation of mine tailings at Palabora Copper. Variables were height, stem length, root length, number of leaves, fresh mass and dry mass. The response of growth in the assorted grass species to the applied treatments would clarify the effect that the various plant growth promoting substances have on the species. Therefore the aim of the study was to determine if the application of PGPS on a selection of grass species would improve the growth and establishment for the revegetation of mine tailings utilised by Palabora Copper.

Due to the nature of the outside trials, environmental factors had an impact on the responses of the grasses to the applied treatments. Therefore, it is not clear whether the treatments, or the overriding-environment influences impact seedling growth. These outside trials indicated no statistically significant responses in measured plant growth variables to the weekly-applied PGPS treatments. Furthermore, these initial trials revealed inconsistent and often opposing responses between the summer and winter repeats.

The effects of smoke-water as a treatment have been shown to stimulate germination in seeds (Soós *et al*., 2010), and post-germination studies have shown to increase seedling vigour in response to smoke-water (Soós *et al*., 2009b). Despite this, no statistically significant responses to its application to the grass seedlings was observed. Some instances of significant responses to smoke-water application relative to non-treated controls were witnessed. However, these results were not reproducible, and a benefit to its application could not be inferred. The application of smoke-water resulted in an increase in leaf number for *C. ciliaris* (Appendix Figure 9c and d), but a decrease in stem length, root length, and dry mass was observed in *C. gayana* during the summer trial period (Figure 3.3b, c, and f). Smoke-water application during the winter trial decreased fresh and dry mass for *C. gayana* (Figure 3.3k and l). Most of the grass seedlings treated with smoke-water did not survive the trial period. However, Chumpookam *et al*. (2012) observed significant increases in root lengths of *Pennisetum clandestinum* seedlings after the application of smoke-water to enhance its phytoextraction capacity. Additionally Okem *et al*. (2015) observed significant increases in shoot and root elongation when papaya (*Carica papaya*) transplanted seedlings are exposed to smoke-water treatments. Kulkarni *et al*. (2010) found that onion plants supplied with smoke-water solutions exhibited significantly greater number of leaves, which supports the increased leaf number observed in *C. ciliaris* during the summer trial*;* however, this result was not reproducible during the winter trial.

The effect of exogenous application of the synthetic strigolactone analogue, GR24, has been investigated on *Arabidopsis thaliana* plants to elucidate their effect on plant physiology (Stirnberg *et al*., 2002). No statistically significant responses were observed for GR24-treated seedlings relative to non-treated controls during the summer trial. *Panicum maximum* seedlings that received GR24 were observed to have decreased stem lengths, root length, fresh mass and dry mass in the winter trial period. Additionally, in the winter trial period, *C. gayana* seedlings treated with GR24 were observed to have decreased height, stem length, number of leaves, fresh mass and dry mass relative to non-treated controls. It has been noted that plants utilize strigolactones to adjust their shoot architecture to the changes in environmental conditions (Ruyter-Spira *et al*., 2011). The observed decreases in above-ground measured variables did not necessarily equate to an increase in root length in response to GR24-treatments. Strigolactone have also been shown to affect root-hair elongation and alter root architecture (Koltai, 2011). The application of the synthetic strigolactone, GR24, has a stimulatory effect on lateral root development (Ruyter-Spira *et al*., 2011), but was not observed in this study. In low phosphate conditions, increased strigolactone exudation has been observed by Brewer *et al*., (2012), which results in branch repression, and enhanced lateral root and root hairs. Exogenous application of GR24 has been shown to induce root elongation (Koltai *et al*., 2010), which was also not observed. Therefore the addition of exogenous GR24 to promote plant growth despite detrimental environmental conditions is not supported based on the results observed here from the first set of trials.

As previous research has affirmed a growth promoting effect of 5 nM lumichrome (Matiru and Dakora, 2005b), this concentration was used in the study. Lumichrome application have been shown to stimulate growth in tomato and lotus plants (Gouws *et al*., 2012). The supply of 5 nM lumichrome to roots of soybean and cowpea seedlings elicited early initiation of trifoliate leaf development, and expansion of unifoliate and trifoliate leaves, as well as an increase in stem elongation, which together resulted in an increase in shoot and plant total biomass relative to controls (Matiru and Dakora, 2005b). In monocotyledonous species such as sorghum and maize, the application of 5 nM lumichrome induced leaf area expansion, thereby increasing shoot and total biomass, whilst no effect on the leaf area of some cereals were observed. Additional developmental changes were observed in sorghum, millet, lotus and tomato seedlings with an increase in root growth (Matiru and Dakora, 2005b; Gouws *et al*., 2012) following treatment. No discernible responses to treatment were observed for outside trials in this study. Lumichrome application on grass seedlings during the summer trials increased root length, and fresh mass for *P. maximum*, whereas a decrease in dry mass relative to non-treated controls was observed during the winter trial (Appendix Figure 5c, e, and l). No statistical significance in treatment response to lumichrome was observed in *M. repens* during the summer period, and a decrease in fresh mass relative to non-treated controls were observed in the winter period (Appendix Figure 6k). *Cynodon dactylon* experienced a decrease in number of leaves relative to non-treated plants during the winter (Appendix Figure 7k). No significant responses to any applied treatments were observed in *A. pubescens* seedlings in summer, but during the winter trial an increase in height and stem length were observed (Appendix Figure 8g and h). No statistical significant responses to lumichrome treatment application were observed for *C. cilairis* seedlings in either of the outside trials. *E. teff* was shown to increase in

height, stem length and number of leaves in response to lumichrome during the summer trial, whilst no statistical significance was observed during the winter trials. In the summer trial *C. gayana* responded positively to lumichrome treatments with increased stem length and fresh mass, whilst dry mass decreased in the winter trial.

The applied concentration of CropbioLife™ (CBL), being 5 mL.100L^{-1}, was shown to be beneficial for plants treated with the substance (Dr N Hanekom, Pers. Comm. 2015). The application of CBL has mostly been monitored on crop plants such as apples, tomato, grapes, and maize plants. CropbioLife™ has been found to concentrate flavonoids in the periderm, which assists against wilt disease. Additionally, CBL application on leaves lead to increased root exudates (Dr N Hanekom, Pers. Comm. 2015). CropbioLife™ treatments elicited mostly non-beneficial effects on grass seedlings in this study. Decreases in dry mass were observed in *C. dactylon* during the summer trial (Appendix Figure 7f), whilst no significant response to treatment was observed for this species in the winter period. *Chloris gayana* showed decreases in height, stem and root length as well as dry mass during the summer period (Figure 3.3a, b, c and f), while dry mass also decreased during the winter period (Figure 3.3l). Leaf number was shown to increase, as well as improved health and vigour, in fruit trees (Dr N Hanekom, Pers. Comm. 2015), as well as an increase in fruit size in *Prunus persica* (Hendricks *et al*., 2015). However these positive responses to CropbioLife™ treatments were not observed in the grasses selected for phytostabilisation.

Due to the variations observed between the outside summer and winter trials, to exclude environmental factors the treatments were repeated in the relative controlled conditions of a greenhouse. Given the lack of possible influence from environmental factors, these trial results were combined and statistical analyses conducted. STATISTICA was used to carry out ANOVA analyses and Fisher's LSD *post hoc* tests to determine the significance of differences in measured variables in response to the applied treatments. Smoke-water as a treatment was also eliminated following the lack of significance responses in initial trials, despite some studies indicating that it improves seedling growth (Papenfus *et al*., 2015). The survivability of *M. repens, P. maximum,* and *E. teff* seedling transplants increased during the winter trial period as opposed to the summer trial, which affected the responses to treatments in the measured growth variables (Appendix Figure 5, 6 and 10). This was true for all other grass species utilised in the treatment trials, with more seedlings surviving and being measured at the end of the trial period. Results from the outside trials and the response to the environment and treatments lead to *P. maximum*, *E. teff*, and *M. repens* being eliminated for screening in the greenhouse trials due to their low survivability during the trials. This was unfortunate, given the high germination rates observed for the *E. teff* and *M. repens* species. The addition of AMF (Mycoroot®) as a treatment was also included.

Plants that are able to form symbiotic relationships with arbuscular mycorrhizae (Haslmayr *et al*., 2014), have shown substantially improved plant growth in arsenic-contaminated soils due to the contributions of mixed arbuscular mycorrhizal fungi (AMF) inoculum (Leung *et al*., 2010b). The alleviation of metal phytotoxicity by AMF in soil also helps in sustaining plant survival, by the binding of metals in mycorrhizal structures and the immobilisation of metals in the rhizosphere (Leung *et al*., 2010b). AMF colonisation of plant roots also contributes

to balanced plant mineral nutrition (Estaún *et al*., 1997). The effect on plant growth promotion abilities of colonised grass seedling roots with mycorrhizal inoculum (Mycoroot™) was investigated. No statistical significance was observed for the pooled results of the inside trials in regards to increased growth variables in response to treatment with AMF. Only *C. gayana* displayed an increase in height, number of leaves, and root and stem length, indicating potential benefits to AMF treatments (Figure 3.4a, b, c, and d) for this species. By contrast, *C. dactylon* showed a decrease in height, fresh mass, stem and root length relative to non-treated controls (Figure 3.7a, b, c and e). Longer trial periods would likely indicate a clearer benefit to AMF treatments, as the initial 4-week trial period may not have been sufficient to maximize mycorrhizal colonisation of the transplanted seedlings and subsequently improve plant growth. Mycorrhizal colonisation was assessed microscopically after the 4-week trial period, although mycorrhizal experiments usually run for a minimum of 8-12 weeks (Sitole, 2013). Roots of seedlings were removed after growth variables were measured, to utilise for Trypan-Blue staining. Root colonisation was recorded for all seedlings utilised in the greenhouse trials, aside from *A. pubescens* where no endomycota colonisation could be observed (Figure 4a). No arbuscules were, however, identified during microscopic visualisation. It is possible that, due to the short duration of the growth trials, the AMF were still in the process of colonising the roots of the grasses and had not yet been able to form arbuscules. In future experiments, it may be advisable to allow the plants to grow for a longer period before measurements are taken or colonisation is observed, in order to provide the AMF time to completely colonise their host roots.

The response elicited to lumichrome treatments applied to seedlings in the greenhouse conditions were variable. Predominantly no statistically significant responses in growth variables were observed. Significant responses to lumichrome were only observed in *A. pubescens,* which showed an increase in leaf number (Figure 3.6d). Lumichrome-treated *C. gayana* seedlings had decreased dry mass (Figure 3.4d), which was also observed during the winter trial period (Figure 3.3f). *Cynodon dactylon* seedlings treated with lumichrome had decreased height, stem and root lengths relative to non-treated controls (Figure 3.7a, b and c). Therefore, despite previous research conducted (Matiru and Dakora, 2005b) in the benefits of lumichrome application, no consistent data could be obtained during the trials to indicate potential benefits in its application to improve phytostabilisation purposes.

CropbioLife™ treatment inside the greenhouse, also generated variable responses from the grass seedlings. Only *A. pubescens* seedlings responded positively to CropbioLife™ treatment with increased leaf number relative to non-treated controls, whereas *C. dactylon* seedlings elicited a negative response to CBL treatments with decreased height, stem and root length, and dry mass relative to non-treated controls (Figure 3.7a, b, c and f). The other grass species, *C. ciliaris, E. cenchroides* and *P. setaceum,* seedlings showed no significant response to CropbioLife™ treatments.

Therefore, across all the trials no definite improvement in plant growth could be observed as the results of PGPS application were variable between species and between trials, and were not reproducible. Extending the time-frame of the trials, as well as increasing the sample size of the seedlings in pot trials may yield more consistent results.

The application of *in situ* treatment could yield responses from species native to the Phalaborwa climate, and hence give a more accurate reflection on the effect and industry application of PGPS in the area. A big factor that could also have contributed to inconsistencies in data was transplanting the seedlings from trays to the trial pots. This may have induced stress on the young seedlings, and influence their response to PGPS as well as their survival during the trial period.

4.3 Management / Industry Implications

The use of various grasses in revegetation of mine tailings managed by Palabora Copper has also been employed by other mining companies such as the De Beers Diamond Mines. This company also utilised seed mixes that included *E. cenchroides, P. maximum, M. repens, T. berteronianus, C. ciliaris, C. gayana* and *C. dactylon* (van Rensburg *et al*., 2003). These grasses were utilised by the De Beers Diamond Mines in revegetation of co-disposed tailing dams with the addition of vermicompost application with fertiliser treatments ($CaNO_3$ and $MgNO_3$), whereby *C. ciliaris* and *C. dactylon* were the most successful in pot and field trials. Additionally the rehabilitation of platinum tailings dams of the Impala Platinum Company (Rustenburg, South Africa) and the effect of woodchip waste on vegetation establishment (Rensburg and Morgenthal, 2004) has been previously researched. The company also utilised the same listed grass species for revegetation. Rensburg and Morgenthal (2004) found that the addition of woodchips enhanced vegetation establishment, especially in the early stages of revegetation. Hence the current seed mix utilised by Palabora Copper is generally appropriate for revegetation efforts, and the addition of *E. teff* and *M. repens* would be beneficial, as shown by their high germination rates in the mine capping material. *Melinis repens* has previously been used for revegation purposes (van Rensburg *et al*., 2003; Rensburg and Morgenthal, 2004; Liebenberg and Rensburg, 2013) due to its wide occurance and capacity to grow in all soil types, included distrubed areas (Van Oudtshoorn, 2012). *Aristida adscensiones* and *S. hirtigluma* occurred commonly in the disturbed mine tailing area (Van Oudtshoorn, 2012). The potential of these species in phytostabilisation is of interest, but, both species did not germinate in sufficiently high numbers during the *ex situ* trials to permit PGPS treatment.

Pennisetum setaceum is an exotic grass (Van Oudtshoorn, 2012) that is considered to be invasive, although its predominence in mine tailings would assist rehabiliation of the mine tailings as it requires minimal manipulation to colonise disturbed areas. Its application in phytostabilisation has been utilised to reclaim lead/zinc processing wastes at Kabwe, Zambia (Leteinturier *et al*., 2001), and was recommended for revegetation purposes. AMF colonisation of the roots was also revealed, further aiding in its ability to tolerate mine tailings.

Although the effect of PGPS on a selection of grass species were investigated in this study, the occurance of forbs such as *Sesbania sesban* and *Tephrosia polystachya* at the mine tailings during the site inspections may prove usefull for future investigations. These forbs are beneficial, as they act as soil improvers through nitrogen fixation. Additionally they act as winbreakers to prevent erosion, and supports other plant species (Feedipedia.org, 2015). *S. sesban* has been shown to accumulate heavy metals, therefore it possesses heavy metal tolerance to enhance its

survival in heavy metal contaminated areas (Yang *et al*., 2003). Their role in phytoremediation has also been investigated, whereby it was shown that they are a beneficial pioneering species to alter barren environments, providing organic matter and nutrients such as nitrogen upon their decomposition, in a relatively short time (Chan *et al*., 2003). The rhisophere diversity of *T. polystachya* was investigated through the use of spread plates, and revealed a predominent abundance of the *Bacilli* genera to be present.

Utilisation of the plant growth promoting substances to enhance germination capabilities and seedling survival could be implemented in a synergistic manner. By dipping grass seeds in smoke-water before seeding of tailings would promote germination by breaking seed dormancy (Soós *et al*., 2009a), thereby promoting germination of seeds. Though, these experiments were not conducted in this study, recent research in smoke-water to enhance the phytoremediation potential of *Pennisetum clandestinum* in cadmium contaminated soil was shown to improve the plants' phytoextraction capacity, and positive effects on shoot and dry weight were observed (Okem *et al*., 2015). The mycorrhizal treatments (Mycoroot™) have shown that most of the grass species utilised are capable of being colonised by AMF, which could protect plant roots from heavy metals by providing a physical barrier against the potential uptake of toxic metals (Leyval *et al*., 1997). During the treatment trials, *C. gayana* seedlings utilised in the greenhouse trial responded the most positively towards AMF treatments. This study also revealed the presence of AMF at Palabora Copper, hence the application of Mycoroot™ would supplement the AMF already present *in situ*. The revegetation of tailing dumps with metal tolerant plants and AMF has already been utilised in the industry (Veenendaal *et al*., 1992; Leung *et al*., 2007; Orłowska *et al*., 2011; González-Chávez and Carrillo-González, 2013), and should therefore be considered as a viable application in phytostabilisation of the mine tailings at Palabora Copper. This application would require incorporation of the AMF (Mycoroot™) pellets in the mine tailings before seeding, possibly followed by GR24 treatment. The application of the synthetic strigolactone analogue, GR24, was shown in this investigation to elicit a response in AMF spore germination at very low concentrations (Figure 5.1), which was also revealed in previous research by Akiyama *et al*. (2005). Although, no observable benefits in the application of GR24 to improve plant growth were noted in this study, other applications are still meritable. GR24 application to the mine tailings that have been seeded by grass seeds and ameliorated with AMF pellets (Mycoroot™), would induce hyphal branching in AMF and initiate colonisation of roots. Further steps could be taken to promote plant growth through the addition of lumichrome as a foliar spray, as very low nanomolar concentrations (5 nM) increased number of leaves, height, and root and stem length in some grass species during some of the trials. This low concentration is also capable of eliciting major developmental changes in plants, which include increased stem elongation and leaf area and greater biomass accumulation (Dakora *et al*., 2015).

4.4 Metagenomic DNA Analysis

Metagenomic DNA was isolated from the collected rhizosphere samples and microbial identification was possible, albeit only after enrichment process was applied. Due to difficulties in isolating metagenomic DNA from the sites

using ZR Soil Microbe DNA MiniPrep™, enrichment procedures were implemented. The addition of liquid LB media and GR24 (0.1 µM) lead to success in PCR amplifications with the arbuscular mycorrhizal fungi (AML 1 and 2), and universal bacterial (8F and 1512R) primer sets at their optimized conditions. The addition of GR24 was done to elicit a potential response in arbuscular mycorrhizal fungi that would potentially be present in the sample. Research has shown that the synthetic a strigolactone analogue, GR24, can induce extensive hyphal branching in germinating spores of AMF at very low concentrations.

However, it should be emphasised that enrichment media utilized to culture microbes in a laboratory are inherently selective and therefore only a small, select population of the total microbes in an environmental sample will be able to grow on any given media (Schneegurt *et al*., 2003). Additionally, the manner in which samples are collected, transported and stored prior to DNA extraction influences the microbial flora of the sample. The physical act of sampling itself physically disrupts soil structures in a manner that alters the microbial community. Storing the samples at 4°C for hours or days before extraction can also fundamentally alter the microbial community and results obtained using molecular phylogeny methods will not accurately describe the original native community (Schneegurt *et al*., 2003). Whilst recognising these limitations, these techniques were applied to provide an indication of the microbiome of the mine tailing environment, and whether there are beneficial species present that could be isolated. These isolates could be further utilised in the future to inoculate grass seeds, to assist in their survival for revegetation purposes.

Of the collected samples only two *P. setaceum* rhizosphere Site 2 samples (PennRD1 and PennRD5) and one *S. hirtigluma* rhizosphere Site 2 (StipaRD5) sample produced amplicons after PCR. Whilst *S. hirtigluma* did not produce any AMF amplicons of the 18S rRNA gene after PCR, both of the *P. setaceum* samples did. This would indicate the plants' ability to acquire a symbiotic relationship with AMF present on site. The positive transformants were identified and subjected to restriction digest analysis. The enzymes utilised for this were *Eco*RI, *Hinf*I, *Rsa*I, *Alu*I, and *Nci*I. These enzymes have different restriction recognition sites to optimise the probability at identifying polymorphisms in the PCR produced fragments. The enzymes were chosen due their ability to recognise short sequences on the amplified DNA and cut at the amplicon relatively frequently. Therefore various sizes of DNA fragments will be produced after restriction digests of the amplicons produced by the primer pairs that amplify AMF and bacterial sequences, 800 bp and 1500 bp respectfully. When the plasmids containing different sequences were isolated, sequenced and subsequently subjected to BLAST, the results indicated the presence of at least two glomeromycota variants (Uncultured *Glomus* partial 18S rRNA gene, isolate Peraza cay, clone PE4-23T, uncultured Glomeromycota clone K1_3 18S ribosomal RNA gene, partial sequence) present on site (Table 4.1). This conforms with previous research at sites which would be detrimental for sustaining plant life, whereby AMF symbiosis assists in the acquisition of nutrients in nutrient poor environments (Tonin *et al*., 2001), and improves plant tolerance to heavy metal stress in polluted soils (Leyval *et al*., 1997). However, some of these sequences retrieved from sequencing that were subjected to BLAST nucleotide searches, returned sequences with the same accession numbers, despite clear variations in the DNA fragments produced from restriction digests. This is a

limitation with this type of metagenomics analysis, in that only very limited sequence data is available and isolates can only be reasonably identified to the genus level.

The microbial diversity that was assessed with the 8F and 1512R primer set indicated an array of microbial life present on site. This primer set, originally designed by Weisburg *et al*. (1991), and modified by Felske *et al*. (1997) was used to determine the presence of bacterial communities present in the samples collected. These universal primers are specific for highly conserved 16S rRNA regions of bacteria. This is indicated in the amplicons produced from the metagenomic DNA extracted from the enriched tailing samples (Table 4.2). The *Bacillus* genus was predominantly present in these enriched *P. setaceum* and *S. hirtigluma* samples. The genus *Lysinibacillus* (KC160856.1) was found in the *P. setaceum* (PennRD5) and *S. hirtigluma* (StipaRD5). This genus is distinguished from other members of the group due to the presence of aspartate and lysine in the peptidoglycan of the cell wall (Ahmed *et al*., 2007). Various strains have already been isolated from sampled soil in research (Ahmed *et al*., 2007), confirming the isolated sequence of the bacterium is indeed present in soil. The species *L. sphaericus* has been applied for bioremediation of heavy metals, such as cobalt, chromium, copper and lead (Tuzen *et al*., 2007).

The *Burkholderia* bacteria (AP014578.1) found in the *S. hirtigluma* sample is a symbiont of the bean bug *Riptortus pedestris* which is known as a serious pest of leguminous crops. They are present in crypts which are sac-like tissues located at the posterior area of the midgut of the bean bug (Kikuchi *et al*., 2005). The symbiont is acquired every generation from the ambient soil and improves the host's fecundity and growth (Kikuchi *et al*., 2007). Recently, however, eight *Burkholderia* species have been found to elicit effective nodule formation on legume roots which are present in the rhizosphere (Gyaneshwar *et al*., 2011). The symbiosis with legumes increases nitrogen fixation, to allow plants to grow in low fertility soils (Lammel *et al*., 2015) Additionally, *Mimosa* trees have exhibited nodulation in symbiosis with *Burkholderia*, whereby the symbiosis was enhanced by AMF (Lammel *et al*., 2015), where faster growth rates were observed from the synergistic symbiosis.

The *B. cereus* strain that was isolated from the *S. hirtigluma* sample indicates its presence in the rhizosphere. This bacterium is a well-known food poisoning agent. This bacterium has been isolated from various environmental niches, ranging from manured soils to arthropod guts. Their presence in the rhizosphere of plants are thought to be as antagonistic against fungal species (Pandey *et al*., 2001)**.** Studies have also revealed that among soil bacteria, *B. cereus* are able to attach to AMF hyphae (Scheublin *et al*., 2010). The use of *B. cereus* to alleviate metal toxicity in plant has been investigated. Microbial inoculants of heavy metal adapted *B.* cereus or *Candida parapsilosis* showed a bioremediation potential and helped to improve plant development in heavy metal contaminated environments (Azcón *et al*., 2010).

Among the other *Bacillus* detected was *B. anthracis* (CP010852.1) which was found in the *S. hirtigluma*. This notable pathogen, the causative agent of anthrax, is commonly found in soils worldwide, where its survival is crucial for initiating subsequent anthrax epidemics (Smith *et al*., 2000). Research conducted by Smith *et al*. (2000) investigated the diversity of *B. anthracis* in the Kruger National Park, and due to the proximity of the Park to the

mine location transmission of these microbes could occur through animal movements. It is most commonly transmitted through ingestion by herbivores whilst grazing or browsing. Soil is the main habitat of endospore-forming, aerobic bacilli, indicated by the presence of the majority of sequences generated from all the samples.

The two identical sequences (HM131955.1) isolated from the PennRD5 sample matched a sequence of a soil bacterium that was generated by Huang and Tan (unpublished) from banana farm soil, currently. The isolated sequence (FM873623.1) from the *S. hirtigluma* sample was identified to be a member of the *Paracraurococcus* genus (Täubel *et al*., 2009). The genus *Paracraurococcus* has been previously isolated from soil (Saitoh *et al*., 1998), were the bacterium is facultatively photoheterotrophic and capable of reducing nitrate to nitrite, although denitrification was not observed. The other sequence (FJ479270.1) matched that of sequences generated in a study by Youssef *et al*., 2009, although no specific genera could be identified due to limited information regarding the sequence in GenBank.

4.5 Culturable Bacteria Analysis

To investigate culturable bacteria from the collected mine tailings, a dilution series of rhizosphere samples collected from the tailing site was spread out on LB agar plates and bacterial growth was observed (Figure 7.1). However, the primer set failed to produce amplicons for all the bacterial cultures isolated from the dilution series spread plates (1000x). Thus some isolates have yet to be identified. Three out of the four bacterial culture colonies of the open tailing plate (1000x) that were able to produce amplicons from PCR using the universal bacterial primer set, revealed three cultures of bacteria *Brevibacterium halotolerans* (JX644589.1), *FictiBacillus barbaricus* (KJ575018.1), and an uncultured *Firmicutes* (GQ249508.1) (Table 5.1). Studies on *Br. halotolerans* and its ability to increase the availability of water soluble Cu, Cr, Pb, and Zn in soils and their effect on metal uptake by *Zea mays* and *Sorghum bicolor* have been conducted (Abou-Shanab *et al*., 2008). Researchers indicated that the highest concentrations of Pb (0.2 g kg^{-1}), Zn (4 g kg^{-1}) and Cu (2 g kg^{-1}) were accumulated in shoots of *Z. mays* grown on Cu-rich soil inoculated with *Br. halotolerans*. Bacteria therefore play an important role in the increase of metal availability in soil, thereby enhancing Cr, Pb, Zn, and Cu accumulation by *Z. mays* and *S. bicolor*. Application of bacterial isolates could also be beneficial towards the phytoremediation of heavy metal contaminated sites.

The sequence that matched *F. barbaricus* was from unpublished sequence data generated by Vishnu *et al*. (2014), regarding the taxonomy and characterization of bacteria isolated from deep sea sediments of Bay of Bengal and Andaman Sea. The species is a relatively new species, first described in 2003 (Täubel *et al*., 2003), though currently its role in the rhizosphere is not yet characterized. However, as the sequence was the only close match it could not be definitely concluded to be the *F. barbaricus* species, but would at least belong to the *Fictibacillus* genus. The uncultured Firmicutes bacterium clone (GQ249508.1), is another sequence that matched with unpublished data from Koechling *et al*. (2009) from an investigation of the microbial community composition of anaerobic marine sediments in the Bay of Cadiz. Firmicutes are a phylum of bacteria which can be divided into anaerobic Clostridia, obligate or facultative aerobic Bacilli, and Mollicutes (Fierer *et al*., 2007). Firmicutes are

widely present in soil (Fierer *et al*., 2007), and common in extreme environments, often found to be dominant in alkaline environments (Keshri *et al*., 2013). They have also been isolated from uranium mine tailings (Khan *et al*., 2013).

Bacterial strains isolated from the *E. cenchroides* resulted in only three bacterial cultures that could be amplified by PCR. One of isolates genotyped to match *Bacillus licheniformis (*NR_074923.1). The complete genome sequence of *B. licheniformis* was produced by Rey *et al*. (2004). This bacterium is a Gram-positive, spore-forming soil bacterium that is used in biotechnology industries to create enzymes, antibiotic, biochemical and consumer products. Some isolates have been found to mitigate the effects of fungal pathogens on maize, grasses and vegetable crops (Neyra *et al*., 1999). The uncultured bacterial (AB636955.1) sequence that matched with colony 2 of the pure cultures for *E. cenchroides* was generated by Yamaguchi *et al*. (2011) for the phylogenetic diversity of 16S rRNA from uncultured bacteria. The last of the *E. cenchroides* strains that were able to produce an amplicon, matched sequences generated by Gao (2014, unpublished data) of an uncultured *Bacillus* clone (KM873110.1).

From the *T. polystachya* tailing pure culture plates, the amplicons produced from colony PCRs showed a majority of *Bacillus* strains. Both colonies 2, and 4 matched *Bacillus* sequences from unpublished data. Colony 2 matched the sequence of the *Bacillus* sp. S12206 (KF956662.1), produced by Suyal *et al*. (2013). Colony 4 was matched with *Bacillus* sp. JY01 (EU798946.1) from Jiang *et al*. (2008) unpublished sequence data. Two of the amplicons produced from colony PCR from the purified cultures (colony 6 and 8) showed sequence similarity to *Bacillus vietnamensis* (JQ799107.1). This strain of *Bacillus* was proposed as a species by Noguchi *et al*. (2004), when it was first isolated from fermented fish sauce. The ubiquitous spread of *Bacillus* as a group is profound in that its members can be found in numerous places. Aside from the majority of *Bacillus* that were cultured from the diluted tailing samples, an amplicon sequence that was produced from colony 7 matched with a partial sequence of an uncultured actinobacterium clone (JN037883.1). This sequence was produced by Keshri *et al*. (2013), from an investigation of the population indices of bacteria and archaea from saline-alkaline soil and a possible microbe-environment pattern that was established using gene targeted metagenomics. The role of actinobacteria isolates for heavy metal tolerance for bioremediation capabilities have been investigated by Hema *et al*. (2014). Actinobacteria are widely distributed in terrestrial and aquatic ecosystems, especially in soil, where they have a crucial role in recycling refractory biomaterials by decomposition and humus formation (Ventura *et al*., 2007).

The *C. ciliaris* pure culture plates produced some of the more diverse sequence amplicons, indicating the most variable cultured bacteria. Colony 1 and 5 generated amplicon sequence that was similar to a *Cupriavidus* sequence (LC065169.1) present in GenBank. This sequence was produced by Amin *et al* (2015, unpublished). The genus *Cupriavidus* is commonly found in the soil (Vandamme, 2004). An isolate of this genera is known for its tolerance of heavy metals. *Cupriavidus necator* is highly resistant to copper and growth initiation is strongly stimulated by copper (Makkar and Casida, 1987). The pure culture of colony 2 was shown to match the sequence of an uncultured

Arthrobacter species clone (JQ793579.1) that was generated by Yousuf *et al*. (2012). *Arthrobacter* has a ubiquitous presence in soil. It has been found that based on the degree of pollution the dominance of *Arthrobacter* increases to become a dominant genera present in heavily metal-polluted soils (Hong *et al*., 2015). This group of bacteria is well known for its ability to metabolise numerous substances. An *Arthrobacter* species has been isolated from long-term tannery waste contaminated soil, and shown to be tolerant to hexavalent chromium [Cr(VI)] and have the ability to reduce Cr(VI) to Cr(III), which is a less toxic substance (Megharaj *et al*., 2003). Therefore the role of *Arthrobacter* in bioremediation of heavy metal contaminated mine tailings could prove beneficial.

The amplicon for colony 4 4 of *C. ciliaris*, was identified as a *Bacillus* species (EU685816.1), whose sequence was from unpublished data of Khanna *et al*., (2008). Based on the generated amplicon sequence for colony 6, BLAST results showed that it matched with a sequence for a strain of *Paracoccus siganidrum* (KM015451.1), generated by Shrivastava (2014) from unpublished data. *P. siganidrum* was first isolated from the gastrointestinal tract of fish (Liu *et al*., 2013). The *Paracoccus* genus is comprised of metabolically versatile organisms that have diverse degradative capabilities. The *P. denitrificans* species is capable of thriving in soil under anaerobic or aerobic conditions. Characteristic of this bacterium is its ability to convert nitrate to dinitrogen through denitrification (Baumann *et al*., 1996). A novel isolate of *P. denitrificans* was found in a coal mine from India, that was capable of utilizing N, N-dimethylformamide (DMF) as a carbon and nitrogen source. Therefore this bacterium could be beneficial to bioremediation efforts.

The final culture that was identified through amplicon sequencing, matched unpublished sequence data of a *Roseomonas* strain (LN810637.2) generated by Ramaprasad *et al.* (2015). The *Roseomonas* genera have been isolated from various environments, and a strain has been isolated from the soil which is capable of degrading triazophos (Chen *et al*., 2014). *Roseomonas* was also included in a selected consortium for crude oil-degradation to aid in bioremediation efforts of soil contaminated with crude oil, the consortium enhanced to removal efficiency of crude oil (Zhao *et al*., 2011). Therefore investigating the potential use of *Roseomonas* in bioremediation of heavy metal mine tailings might elucidate beneficial results.

Overall, bacterial isolates that were culturable and could be identified through molecular techniques were shown to be predominantly members of the *Bacillus* genus. Considering the number of clones generated which did not yield amplicons during PCR analysis, several unidentified bacterial isolates from the streakeded rhizosphere tailing samples still remain. Further analysis should be conducted to identify these unknown isolates.

5. Conclusion

This research was undertaken to determine if the application of a number of PGPS would aid in the establishment and survival of a number of grass species in a heavy metal contaminated environment, thereby enhancing the revegetation of mine tailings. This was achieved through utilising pot treatment trials and the application of lumichrome, CropbioLife™, GR24, smoke-water and arbuscular mycorrhizal fungi (Mycoroot™) as PGPS treatments. Seedlings of the selected grass species were grown on commercial vermiculite in trays for two weeks, before transplanting the seedlings to pots containing capping material collected from Palabora Copper. Variables were measured to determine the possible advantageous effect that PGPS would have on the chosen grass species to assist in their survival to perform phytostabilisation on site. No net positive affect of PGPS application to grass seedlings could be observed. Aside from *Chloris gayana* eliciting increased height, stem length, root length and leaf number in response to AMF treatments during the greenhouse trials, the remaining grass species tested did not invoke similar statistically significant positive responses to the application of PGPS treatments. Therefore, based on the nature of these *ex situ* trials, no statistical significant responses to PGPS treatments could be observed to merit the application of PGPS to improve on current rehabilitation techniques used by Palabora Copper on mine tailings. However, as AMF was shown to be present *in situ*, it is suggested that these could be supplemented with additional Mycoroot® AMF to improve plant growth as indicated with the responses elicited by *C. gayana*. Longer trial periods could potentially elicit greater positive responses in other grass species.

However, as the trials were carried out *ex situ*, over a limited period and with the added stress of transplanting seedlings to pots, results in response to treatments may not reflect results had trials been carried out *in situ*. Extending the trial periods and increasing the sample size for treatments may yield more beneficial and conclusive responses to treatments. Additionally, to avoid transplant stress, grass seeds could be cultivated directly on capped mine material *in situ.*

During the germination trials, it was generally observed that more grass seeds germinated in the vermiculite capping material than seeds present in the potting soil. Thus, it would be recommended to add *E. teff* and *M. repens* to the grass seed mix utilised by the company to revegetate the area, given their propensity to germinate in the capping material. However, these species were not utilised during the greenhouse trials and therefore their response to AMF and the other PGPS could not be determined.

The utilisation of the universal bacterial primer pair (F8 and 1512R) proved useful in generating amplicons from the DNA extracted from the samples, and from the bacterial spread plates. However due to enrichment techniques, bacterial cultures identified from these procedures are not fully indicative of the diversity of the microflora of the environment where sampling was conducted. As a consequence, the diversity of the microbiome was limited to an assortment of bacterial strains capable of growing in certain conditions, additionally sampling and storage methods would alter the diversity of the microflora. Of the bacterial cultures that were identified to genus level, further research is required to fully identify them as potential plant growth promoting bacteria, which could be of benefit

for rehabilitation of mine tailings. The bacterial isolates identified through the enrichment techniques revealed *Bacillus* to be predominantly present in the rhizosphere samples collected. Additional bacterial genera that are potentially beneficial towards bioremediation practices, such as *Lysinibacillus* and *Burkholderia*, were identified from metagenomic DNA extractions. Additionally, spread plates analysis identified further beneficial bacteria such as *Brevibacterium, Cupriavidus, Arthrobacter, Paracoccus* and *Roseomonas* for bioremediation purposes based on their occurrence and tolerance in heavy metal environments.

If additional research into the microbial diversity of the Palabora Copper mine site is desired, the use of EcoPlates™ (Biolog) could potentially provide more information regarding the impact of environmental change on microbial communities. These plates were created for microbial community study and contain 31 of the most widely used carbon sources, whereby the community of organisms will yield characteristic reaction patterns, termed as a metabolic fingerprint (Stefanowicz, 2006).

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7. Appendices

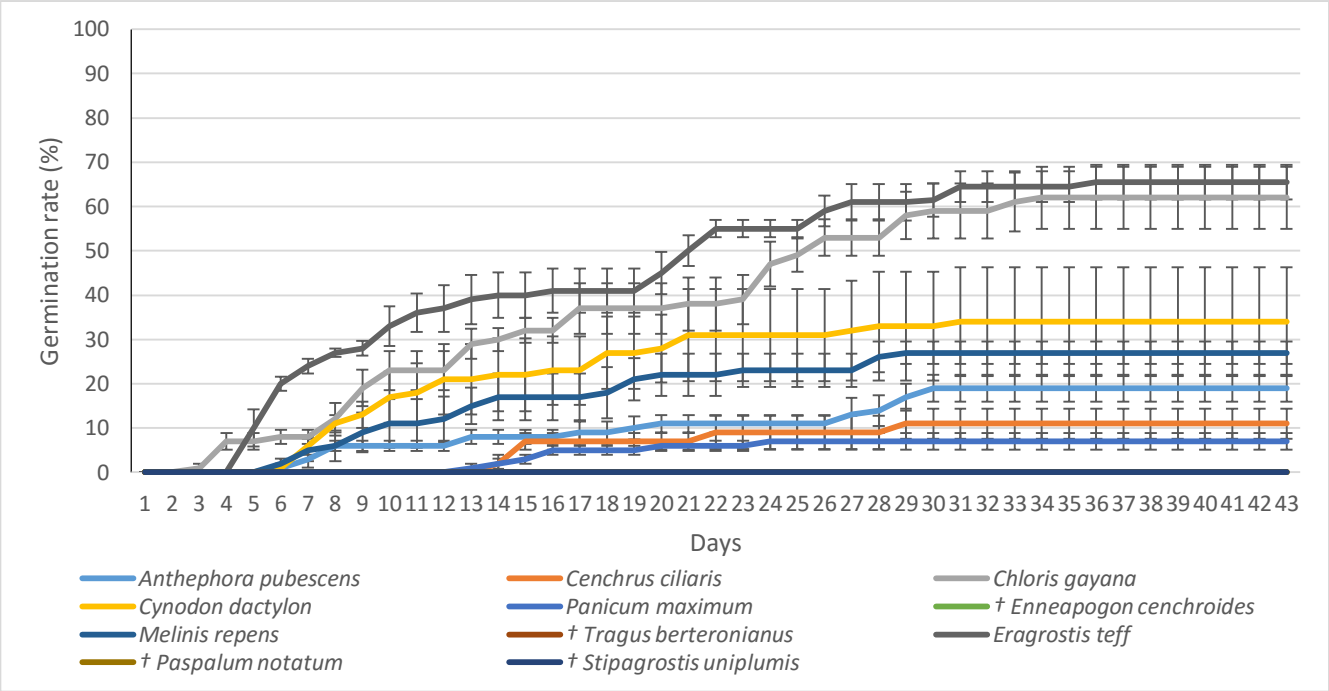

Figure 1: Germination rate for seed of commercial grass species available to Palarabora Copper for use in mine dump rehabilitation. Seeds were germinated outside the IPB department, Stellenbosch University, on commercial potting soil, with daily watering during the winter. †=unresponsive species.

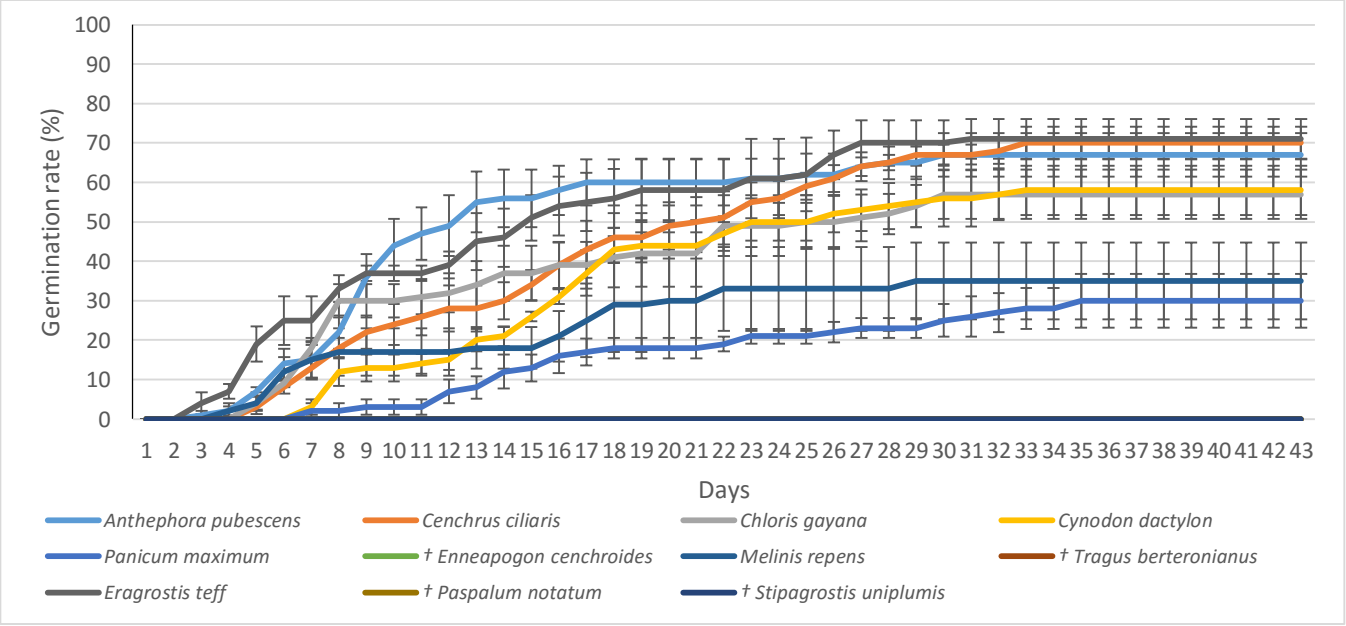

Figure 2: Germination rate for seed of commercial grass species available to Palarabora Copper for use in mine dump rehabilitation. Seeds were germinated outside the IPB department, Stellenbosch University, on mine capping material used to cover the tailing dumps, with daily watering during the winter. †=unresponsive species.

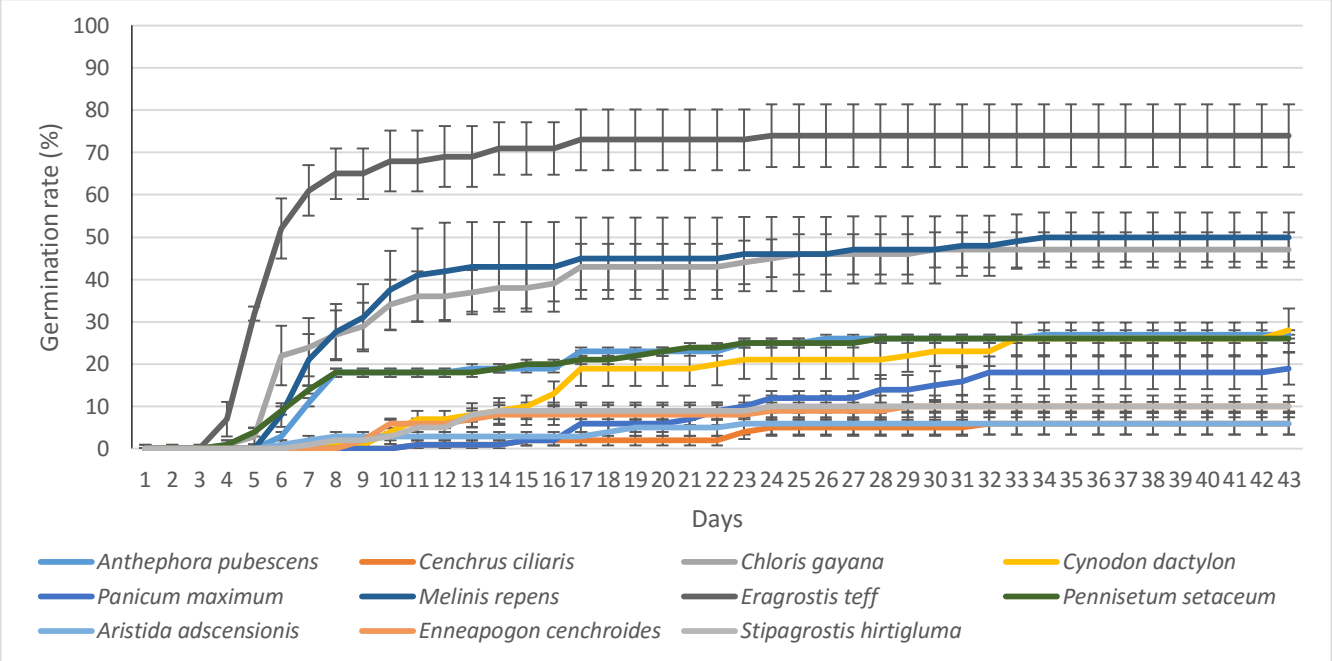

Figure 3: Germination rate for seed of commercial grass species available to Palabora Copper for use in mine dump rehabilitation, as well as seed of locally dominant species occurring on site. Seeds were germinated under greenhouse conditions on commercial potting soil, with daily watering during the summer.

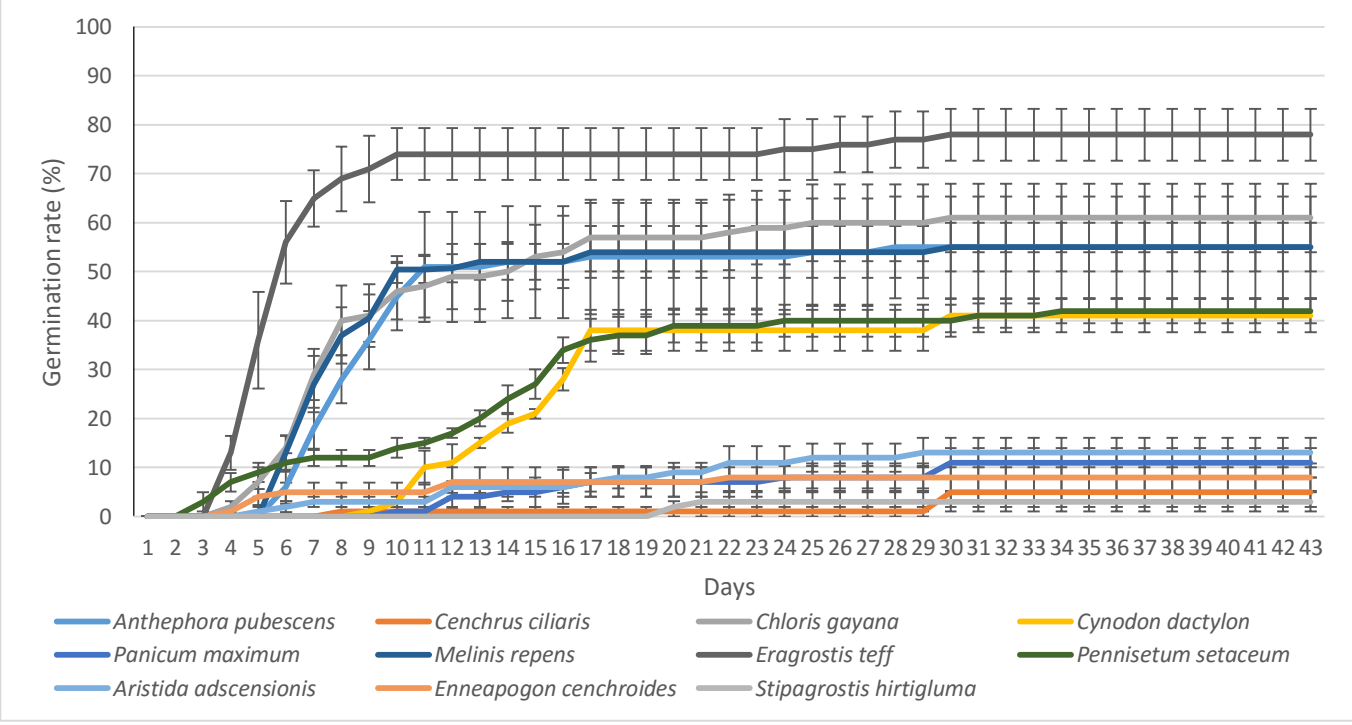

Figure 4: Germination rate for seed of commercial grass species available to Palabora Copper for use in mine dump rehabilitation, as well as seed of locally dominant species occurring on site. Seeds were germinated under greenhouse conditions on mine capping material used to cover the tailing dumps, with daily watering during the summer.

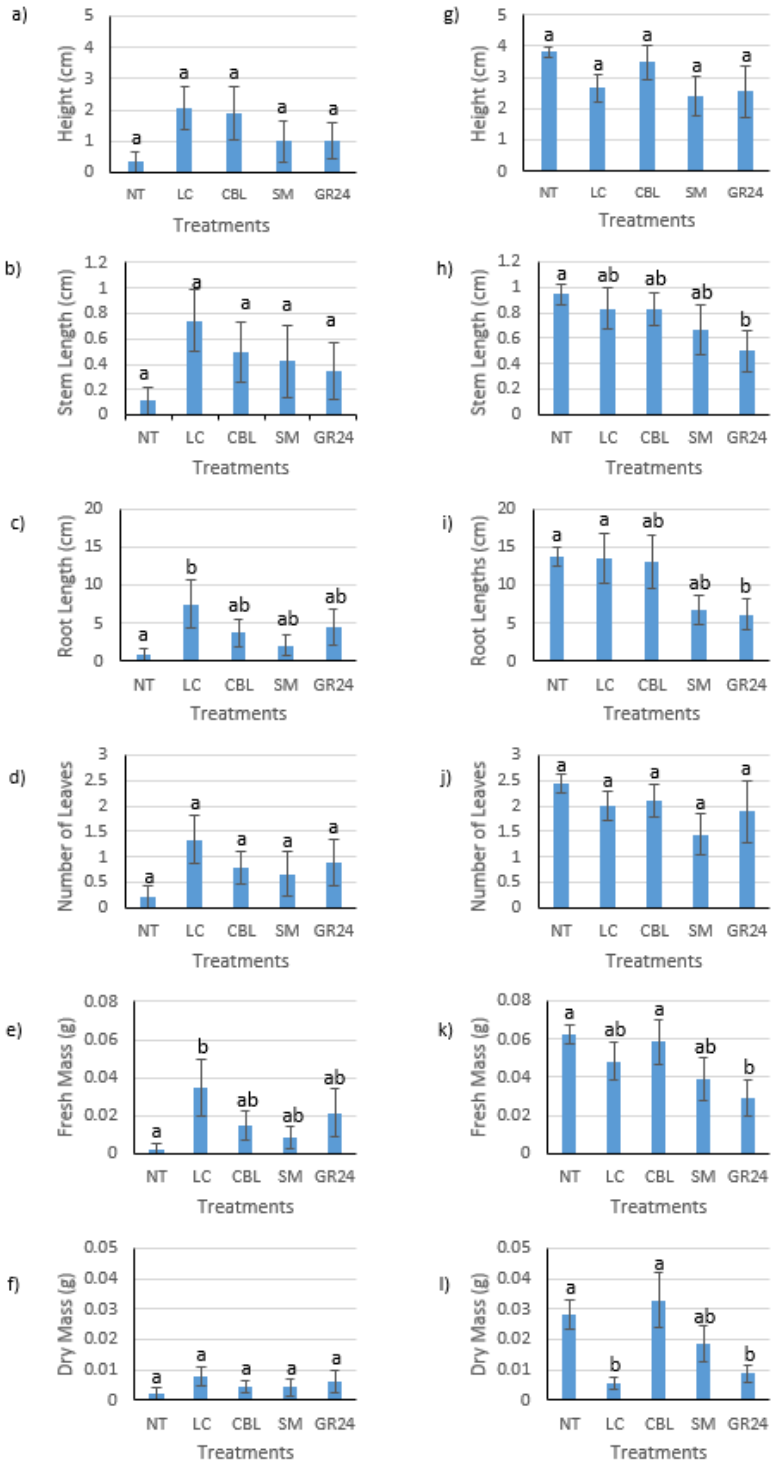

Figure 5: Growth of *Panicum maximum* seedlings in mine capping material during outside trials held during either the summer months (a-f) or winter months (g-l) of 2015. Parameters measured were plant height (a. g), stem length (b, h), root length (c, i), number of leaves (d, j), fresh mass (e, k) and dry mass (f, l). NT=no treatment (water only), LC=lumichrome, CBL= CropbioLife™, SM= Smoke-water, GR24=synthetic strigolactone. Bars represent the mean of 9 replicates (n=9) ± standard error. Treatments labelled with the same letters within a graph were not significantly different from each other ($p>0.05$).

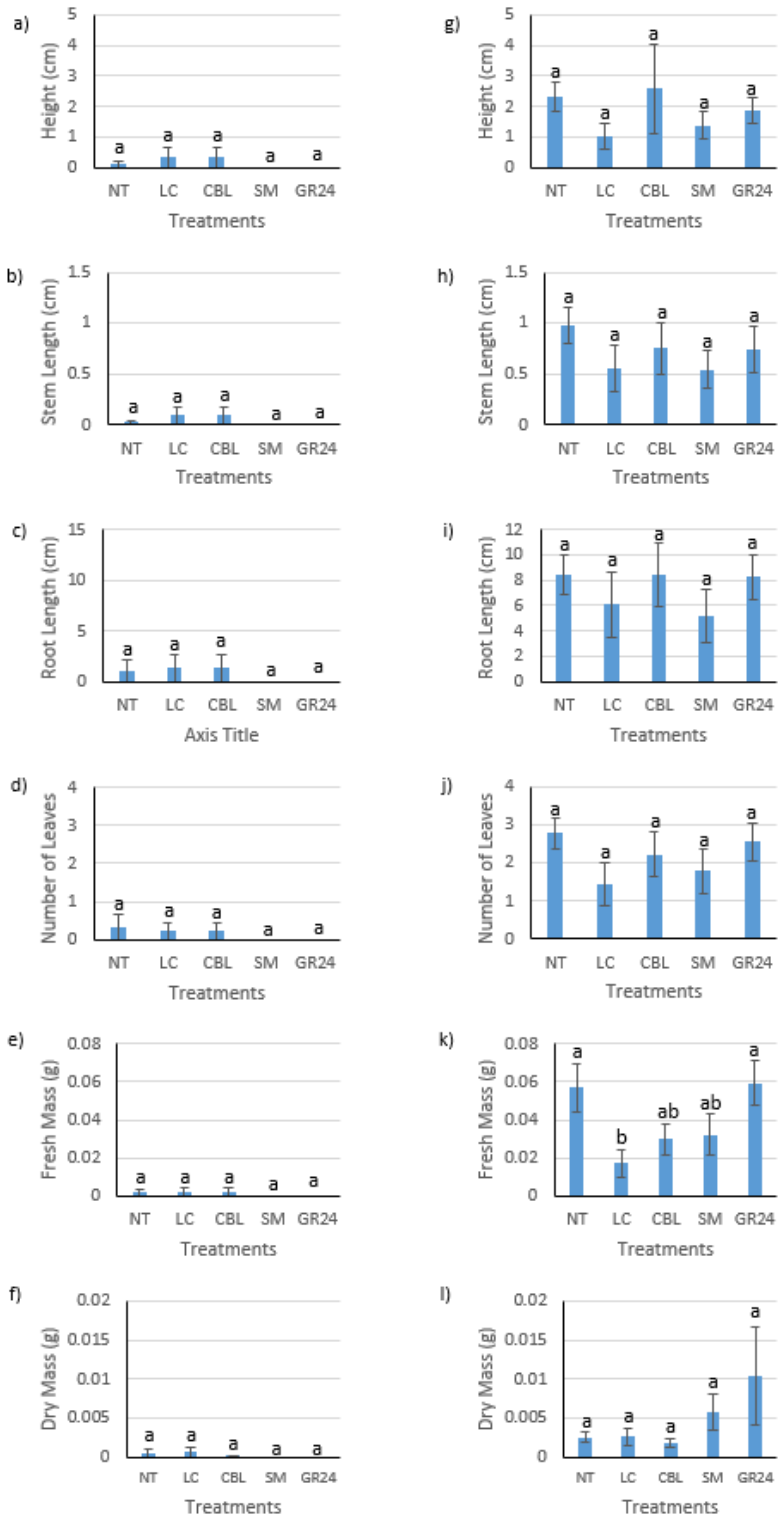

Figure 6: Growth of *Melinis repens* seedlings in mine capping material during outside trials held during either the summer months (a-f) or winter months (g-l) of 2015. Parameters measured were plant height (a. g), stem length (b, h), root length (c, i), number of leaves (d, j), fresh mass (e, k) and dry mass (f, l). NT=no treatment (water only), LC=lumichrome, CBL= CropbioLife™, SM= Smoke-water, GR24=synthetic strigolactone. Bars represent the mean of 9 replicates (n=9) ± standard error. Treatments labelled with the same letters within a graph were not significantly different from each other (p>0.05).

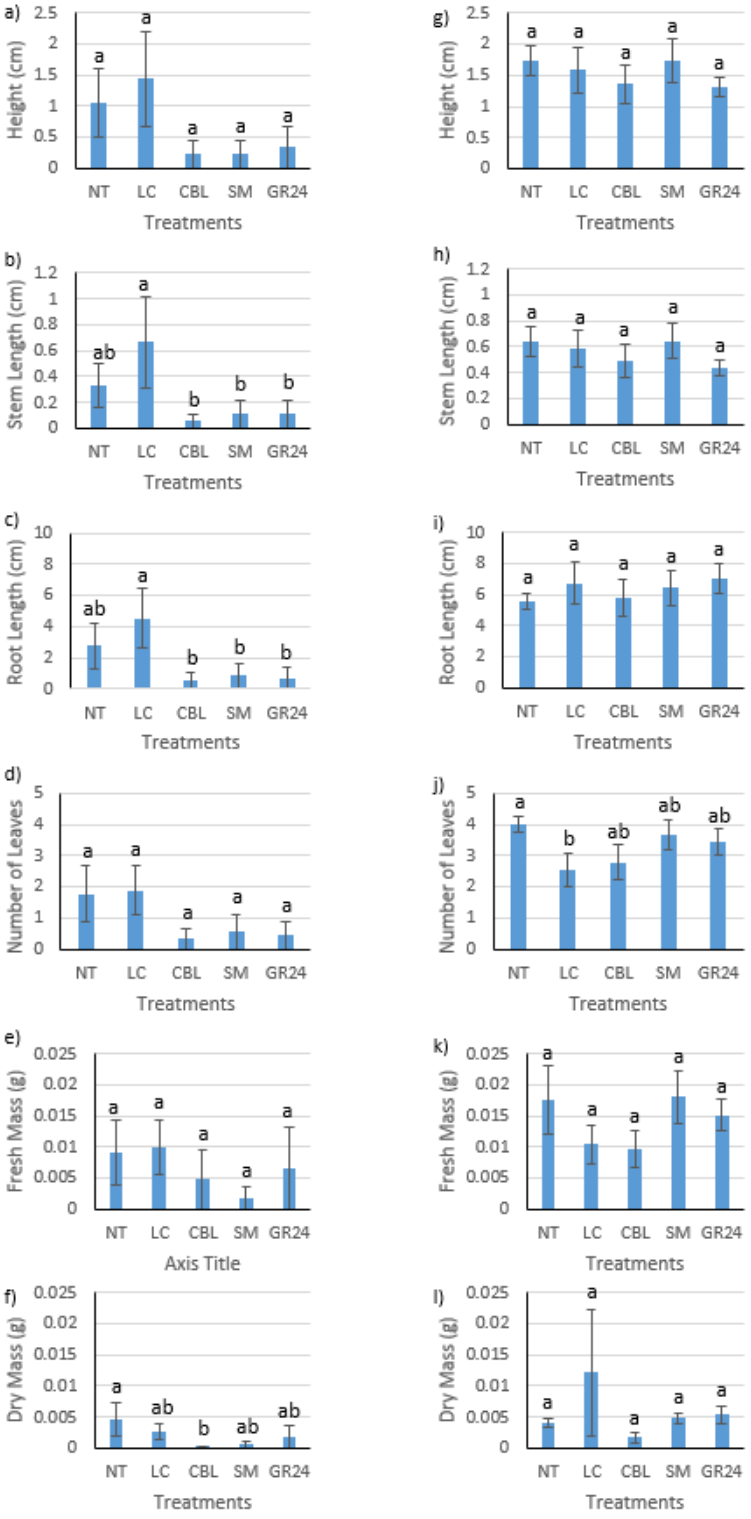

Figure 7: Growth of *Cynodon dactylon* seedlings in mine capping material during outside trials held during either the summer months (a-f) or winter months (g-l) of 2015. Parameters measured were plant height (a. g), stem length (b, h), root length (c, i), number of leaves (d, j), fresh mass (e, k) and dry mass (f, l). NT=no treatment (water only), LC=lumichrome, CBL= CropbioLife™, SM= Smoke-water, GR24=synthetic strigolactone. Bars represent the mean of 9 replicates (n=9) ± standard error. Treatments labelled with the same letters within a graph were not significantly different from each other (p>0.05).

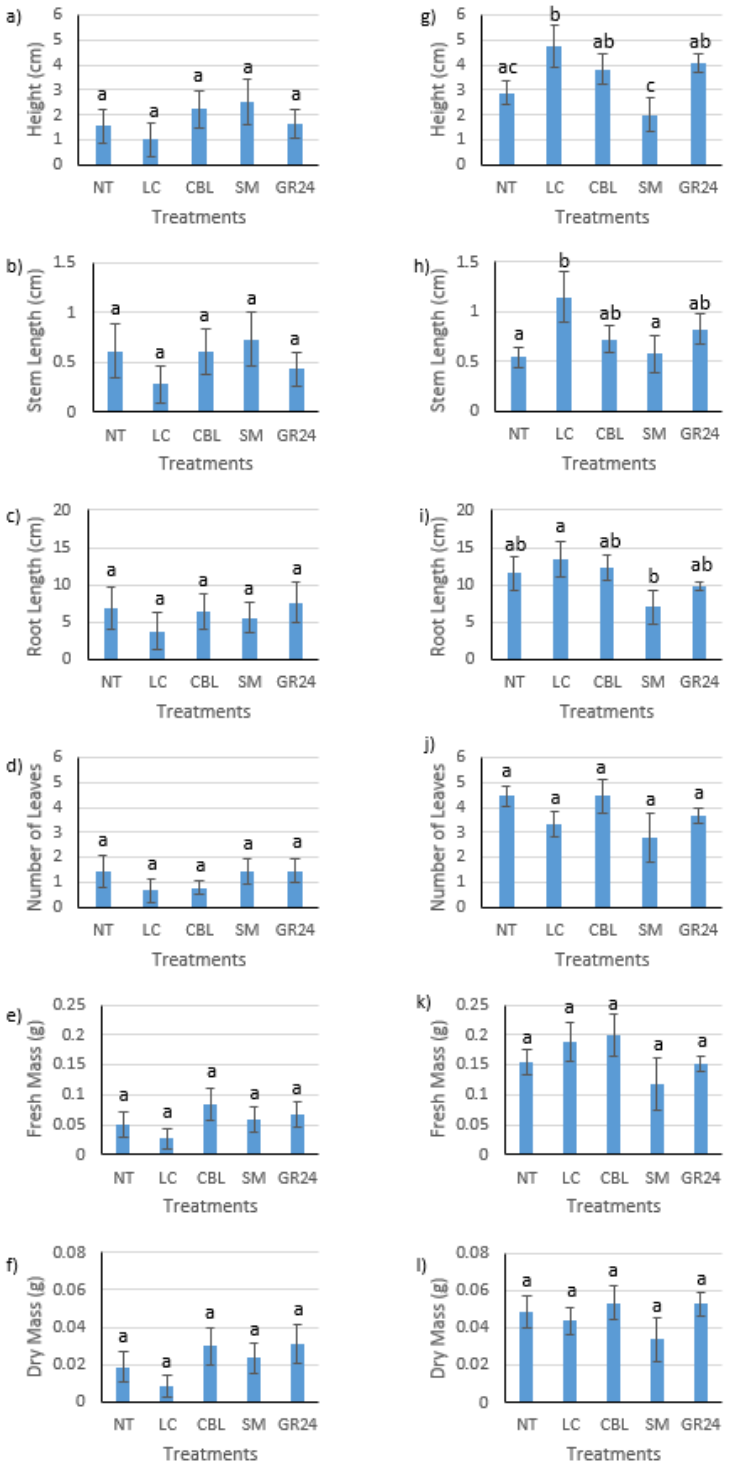

Figure 8: Growth of *Anthephora pubescens* seedlings in mine capping material during outside trials held during either the summer months (a-f) or winter months (g-l) of 2015. Parameters measured were plant height (a. g), stem length (b, h), root length (c, i), number of leaves (d, j), fresh mass (e, k) and dry mass (f, l). NT=no treatment (water only), LC=lumichrome, CBL= CropbioLife™, SM= Smoke-water, GR24=synthetic strigolactone. Bars represent the mean of 9 replicates (n=9) ± standard error. Treatments labelled with the same letters within a graph were not significantly different from each other ($p>0.05$).

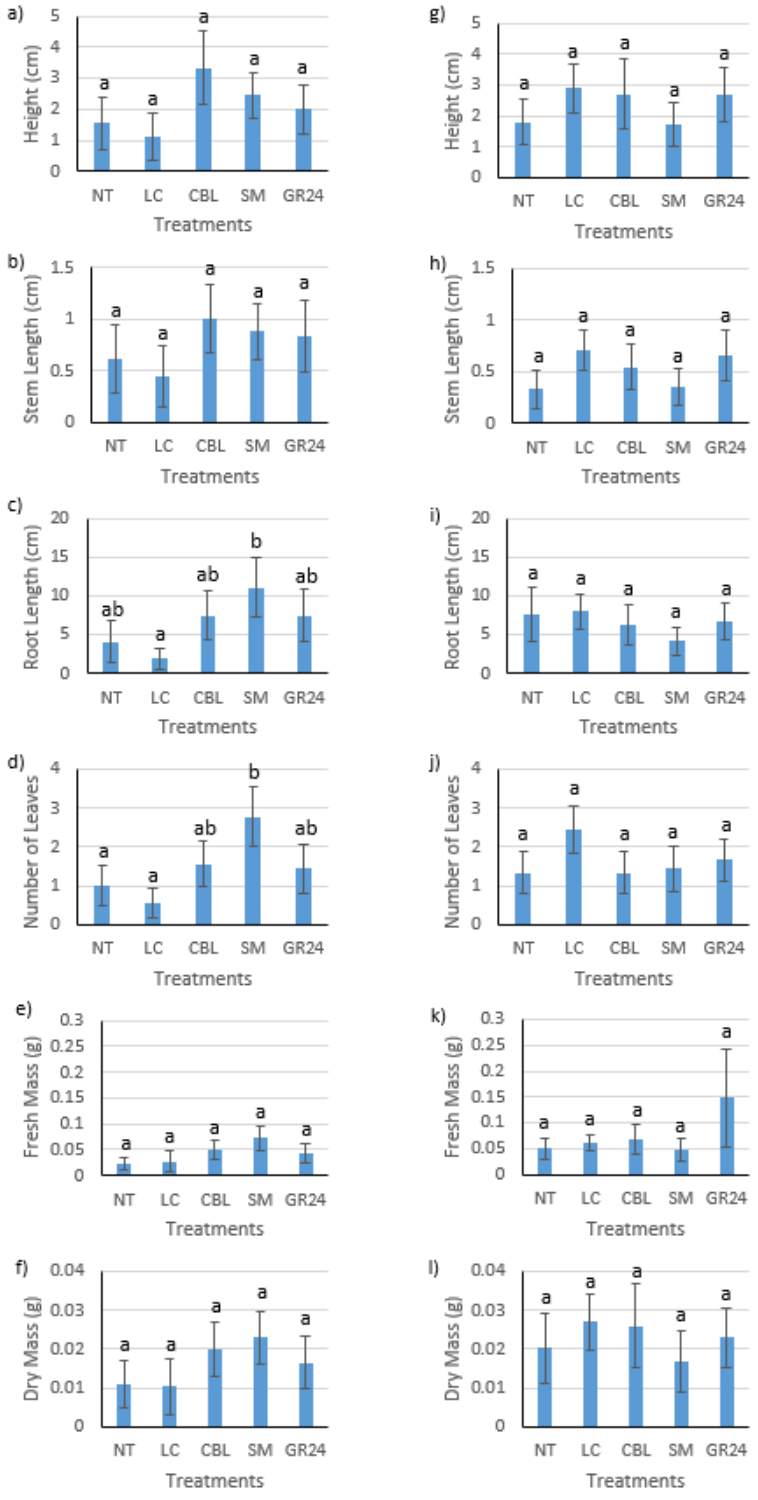

Figure 9: Growth of *Cenchrus ciliaris* seedlings in mine capping material during outside trials held during either the summer months (a-f) or winter months (g-l) of 2015. Parameters measured were plant height (a. g), stem length (b, h), root length (c, i), number of leaves (d, j), fresh mass (e, k) and dry mass (f, l). NT=no treatment (water only), LC=lumichrome, CBL= CropbioLife™, SM= Smoke-water, GR24=synthetic strigolactone. Bars represent the mean of 9 replicates (n=9) ± standard error. Treatments labelled with the same letters within a graph were not significantly different from each other ($p>0.05$).

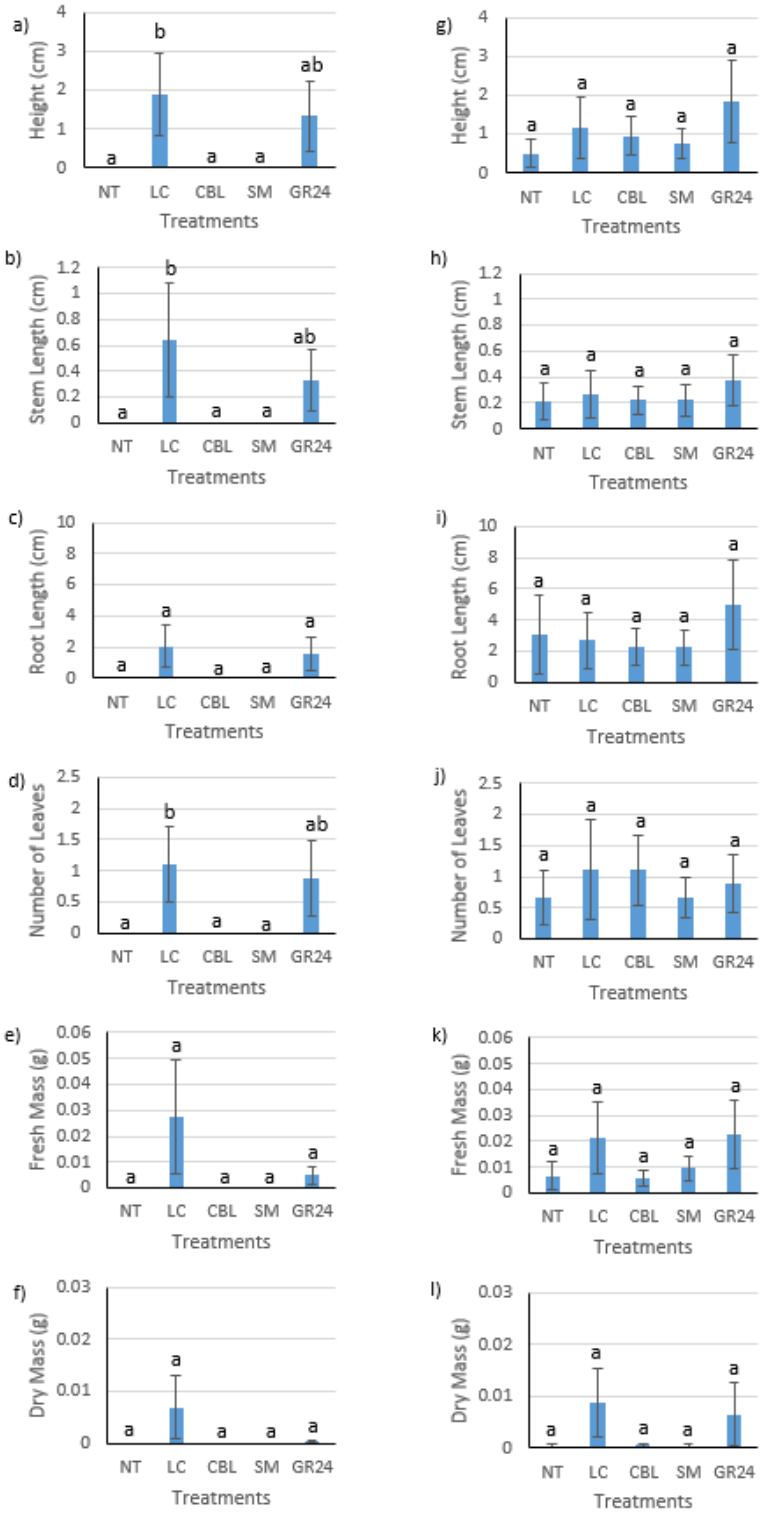

Figure 10: Growth of *Eragrostis teff* seedlings in mine capping material during outside trials held during either the summer months (a-f) or winter months (g-l) of 2015. Parameters measured were plant height (a. g), stem length (b, h), root length (c, i), number of leaves (d, j), fresh mass (e, k) and dry mass (f, l). NT=no treatment (water only), LC=lumichrome, CBL= CropbioLife™, SM= Smoke-water, GR24=synthetic strigolactone. Bars represent the mean of 9 replicates (n=9) ± standard error. Treatments labelled with the same letters within a graph were not significantly different from each other ($p>0.05$).