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FINAL REPORT:

APPLICATION OF PLANT GROWTH PROMOTING SUBSTANCES AND ARBUSCULAR MYCHORRIZAL FUNGI FOR THE PHYTOSTABILIZATION OF MINE DUMPS AND TAILINGS AT PALABORA COPPER

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INTRODUCTION

A short-term rehabilitation study was initiated by SAEON in 2014, in collaboration with the Institute for Plant Biotechnology at the University of Stellenbosch. An MSc student – Mr Jacob Rossouw – was appointed to inform PC phytostabilization efforts by identifying those grass species responsive to growth manipulation; with these being theoretically superior revegetation candidates in the stressed dump/tailings environments where plants must survive salinity, unfavorable pH, low or excessive nutrient concentrations, moisture limitations and heavy metal accumulation. Overarching objectives were to:

i) Establish seed germinability in the vermiculite capping medium for grass species sourced commercially, and for those pioneer species establishing naturally through succession;
ii) Assess possible beneficial effects of a range of growth-promoting treatments on commercially available and *in situ* harvested grass species seed; and
iii) Catalogue the naturally occurring microbiota present in the rhizosphere of selected grass and forb species successfully colonized in areas that were capped two or 10-12 years previously.

Plant hormones and secondary metabolites have been shown to modulate plant responsiveness to environmental stresses, either directly or through synergetic effects such as the induction of beneficial bacterial and mycorrhizal symbioses. Growth–manipulating treatments therefore involved the exogenous application of these compounds.

SYNTHESIS

Capping material and mine tailings were collected and used as the germination and growth medium in pot trials investigating the effect of five classes of plant growth promoting substances (PGPS) on several grass species currently used in efforts to stabilise those areas of Palabora Copper requiring revegetation.

Lumichrome, strigolactones (GR24), flavonoids (CropbioLife™), smoke-water (karrikins) and arbuscular mycorrhizal fungi (Mycoroot™) have all been shown to impart stress-tolerance and improve growth response in plants following artificial

application. These were used as PGPS to investigate growth-promoting / establishment benefits in i) *Anthephora pubescens*, *Cenchrus ciliaris*, *Chloris gayana, Cynodon dactylon*, and *Panicum maximum* – these being the five grass species currently used by Palabora Copper for initiating rehabilitation, and ii) additional grass species – commercially available and harvested *in situ,* with theoretical suitability to the local environment.

PGRS treatments were applied to 2-week old transplanted grass seedlings in pot trials. Trypan-Blue staining procedures were used to ascertain which grass species formed symbiotic relationship with arbuscular mycorrhizal fungi (AMF), which would potentially aid their survival in otherwise inhospitable areas. Germination rates were measured, with *Eragrostis teff* and *Melinis repens* – 2 species not currently used by PC, germinating the quickest in the vermiculite capping material.

Capping material and mine tailing samples were collected at sites under revegetation by PC. 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 *C. ciliaris*, *Enneapogon cenchroides*, and *Tephorisia polystachya* (a locally abundant forb species) at site 1 and *C. ciliaris*, *Stipagrostis hirtigluma*, *T. polystachya,* and *Pennisetum setaceum* at site 2. At both sites the soil of open areas devoid of plants was also sampled as a negative control. Metagenomic DNA was extracted from the collected rhizospheres, 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 genus of bacteria detected in the collected tailing samples was *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 phytostabilization 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.

OUTCOMES

Germination trials

Commercial seed was obtained from Diverse Ecological Solutions (Pty. Ltd.) These include the 5 species presently used by Palabora Copper (*A. pubescens, C. ciliaris, C. gayana, C. dactylon and P. maximum*) and 6 alternative species. The latter were selected according to the following criteria: commercial availability, indigenous, mycorrhizal, perennial and so-called pioneer species. The species selected were *E. cenchroides*, E. teff, M. repens*, Paspalum notatum, Stipagrostis uniplumus and Tragus berteronianus**. Highlighted species* are used elsewhere in revegetation activities in the mining sector.

Germination trials were carried out using nutrient rich potting soil, commercial vermiculite and nutrient poor raw capping material collected from PC, and under ambient and greenhouse conditions in Stellenbosch. Trials were repeated in Jan-Mar, Apr-May, Jun-Jul and Aug-Sept 2015. Germinants (seedlings) from the germination trials were used in subsequent treatments accessing the effect of plant-growth promoting substances on seedling vigour (as an indicator of likely survivorship). Of the 11 species tested, only 7 species germinated; being the 5 species presently used by PC in seeding the mine dumps, as well as *E. teff* and *M. repens*. The remaining species (*T. berteronianus, P. notatum, S. uniplumis* and *E. cenchroides*) did not germinate, despite several attempts and modifications to the protocols.

Unfortunately the results from the germination trials varied largely between repeats. However, *E. teff* and *A. pubescens* consistently showed the most rapid, synchronous and highest % germination. Poor germination was recorded for *P. maximum*; with intermediate responses seen for *Cenchrus, Cynodon Chloris* and *Melinis*.

High levels of mortality were recorded following the transplant of seedlings to pots containing raw capping material for treatment with growth-promoters; especially for *P. maximum, E. teff, M. repens* and *C. dactylon.*

A second germination trial was conducted to assess indigenous grass species dominant in the area viz. *S. hirtigluma, C. ciliaris, P. setaceum, Aristida adscensionis* and *E. cenchroides*. In addition, rhizosphere soil samples were collected from around the roots of *E. cenchroides, C. ciliaris, P. setaceum, S. hirtigluma* and *T. polystachya* – a dominant forb species, growing on recently (2 yr) and previously (+10 yr) capped rock dump slopes. Seed was subjected to the same germination trials as outlined above. Poor germination was recorded for *S. hirtigluma* and *A. adscensionis*, whilst the seed of the remaining 3 species germinated within the range seen for the commercially available seed of the same species

Growth promoters

The role of various commercially available plant growth promoting substances (PGPS) as treatment applications were investigated to determine their effect on growth for the grass species. Lumichrome, strigolactones (GR24), flavonoids (CropbioLife™), smoke-water (karrikins) and arbuscular mycorrhizal fungi (Mycoroot™) were tested as possible growth-promoting additives.

The positive or negative effect of the PGPS, relative to control seedlings treated with distilled water, varied markedly between species, with no single PGPS or species being consistently superior. For example, GR24 increased rooting in *Chloris*, but Lumichrome increased the number of leaves and fresh mass in the same species. Lumichrome increased root length and fresh mass in *P. maximum*, but decreased fresh mass for *M. repens*. Both GR24 and Lumichrome were necessary for the survival of *E. teff* seedlings. Lumichrome increased overall seedling vigour for *Anthephora*, whereas Cropbiolife simply increased leaf number. Treatment with smoke-water decreased rooting in this species. Lumichrome also increased vigour in *Enneapogon*. Mycoroot increased seedling vigour in *Chloris*, but had no effect for *Cenchrus* or *Anthephora*.

For the 5 locally-occurring grass species, the effect of PGPS on the seedlings was similarly highly variable.

Rhizosphere characterization

Microbial DNA was extracted from the rhizospheres of the 5 dominant species present on the rehabilitation sites. Between 2-9 bacterial strains were found in association with their roots. For the most part, the bacterial strains belong to the genera *Baccillus, Brevibacterium* and *Cupriavidus* – all with reported plant benefits (promoting N-fixation and root nodulation, enhancing heavy metal tolerance, increased elemental availability) that would promote survival under stress-conditions. Fungal associates were typically in the *Glomeromycota*, a group of AMF known to assist in the acquisition of nutrients in nutrient poor environments and to improve plant tolerance to heavy metal stress in polluted soils.

For the indigenous grass species, arbuscular mycorrhizal associations were seen to establish in *Cenchrus, Chloris, Pennisetum, Cynodon* and *Enneapogon* during the PGPS trials involving Mycoroot. *Anthephora* was not mychorrizal. However, only *Chloris* seedlings showed increased growth metrics as a result of the association.

Given the unconvincing response of commercial and indigenous, locally dominant grass species to exogenous PGRS application, gene expression in affected plants and the development of a protocol for sensitivity screening were not pursued.

The MSc thesis was submitted for examination in January 2016, and the degree awarded in March 2016 following minor correction.

RECOMMENDATIONS FOR MANAGEMENT

Several findings can be used to improve rehabilitation efforts at PC:

1. The composition of grass species used to initiate revegetation should be reconsidered to include *Eragrsotis teff* and *Melinis repens* and exclude *Panicum maximum*. This is based on the high and synchronous germination, wide tolerance for germination substrates and environmental conditions, and

response to PGPS application, shown by the former two species. *Melinis repens* has previously been used for revegetation purposes.

2. Smoke-water and CropbioLife can be excluded from the list of potential growth promoters, whereas application of Lumichrome should be tested in the field as this PGRS showed positive effects across several species. Additions of lumichrome as a foliar spray, even at very low nanomolar concentrations (5 nM) increased leaf number, height, and root and stem lengths during these trials, making this a promising treatment agent that could increase seedling establishment through increased leaf area and biomass accumulation.
3. Given the theoretical benefits of AMFs (although seen here only in *Chloris gayana*), the capacity for almost all species seedlings to initiate symbioses, and the rich rhizosphere already present for species growing in the revegetated areas, the use of Mycoroot™ is well supported. The revegetation of tailing dumps with metal tolerant plants and AMF has been utilised elsewhere in the industry and should therefore be considered as a viable application in phytostabilization of the mine tailings at Palabora Copper. This application would require incorporation of the AMF (Mycoroot™) pellets into the mine tailings before seeding or into the vermiculite capping layer during seeding to stimulate the formation of a natural rhizosphere, or to substitute until the natural rhizosphere has developed.
4. Strigolactone application, although having little direct effect on seedling survivorship here, could be considered further given its proven role in supporting mycorrhizal associations. Specifically, research has shown that the synthetic a strigolactone analogue, GR24, can induce extensive hyphal branching in germinating spores of AMF at very low concentrations.
5. Metagenomics revealed substantial soil microbiotic communities within the rhizospheres of plant species dominant on dumps capped 10 and 2 years previously, with the probability that survival is enhanced by these symbioses. The bulk of the fungal and bacterial genera isolated are beneficial, having been previously linked to various aspects of plant survival in stressful environments; nodule formation in nitrogen-fixers, bioremediation of heavy metals, the mitigation of fungal pathogens, increased availability of trace elements, and carbon cycling and decomposition.

6. A diverse rhizosphere is likely key in enhancing plant survival under stressful conditions, and can be encouraged or supported by AMF augmentation. EcoPlates™ (Biolog) could potentially provide valuable information regarding establishment of the microbial community. These plates contain 31 of the most widely used carbon sources, upon which a community of microbiota will yield characteristic reaction pattern, or metabolic fingerprint.
7. The germination and PGRS trails here were carried out *ex situ* under climatic conditions vastly different from those experienced at PC, over a limited period of just several weeks per trial, and with the added stress of having to transplant seedlings for subsequent treatment application. Longer periods to more accurately assess the impacts of the PRGS on differential growth is recommended, as is cultivating and treating seeds directly on the vermiculite capping material *in situ*.