MOLECULAR IDENTIFICATION AND CULTURE OF FUNGI NATIVE TO HEAVY METAL CONTAMINATED KAM KOTIA MINE

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Heavy metal (HM) contamination can be caused by human activity through mining, agricultural applications of fertilizer and manufacturing, and can prove detrimental to the health of humans, plants, animals and soil microbes. As environmental regulations are strengthened throughout the developed and developing world, there has been increased industrial and scientific interest in the remediation and restoration of contaminated sites. Conventional remediation strategies require the excavation of the contaminated soil, which is costly, and further damages the soil's structure and microbial community (Mulligan *et al.* 2001, Jankaite and Vasarevičus 2005). Phytoremediation, the use of plants to immobilize, sequester and/or extract heavy metal contaminants is a relatively inexpensive and non-destructive remediation strategy that could allow for more efficient restoration of contaminated land. Recent research has identified plants such as poplar as being ideal candidates for bioremediation projects, and has begun to describe the genetic and physiological basis for phytoremediatory processes in these plants.

The soil microbiome, a diverse community of bacteria, free-living fungi, and fungi that associate symbiotically with plants must also be considered when phytoremediation strategies are planned. Of particular interest to our laboratory are the Arbuscular Mycorrhizal Fungi (AMF). These fungi, members of the division Glomeromycota, are obligately symbiotic with 80% of land plants (Schüßler *et al*. 2001). AMF have been found to enhance the phytoremediatory abilities of plants (Giasson *et al*. 2006). AMF functionally extend plants' root networks, allowing them greater access to water and nutrients (Hetrick *et al*. 1988), and through the production of organic acids and phosphatases the fungi can mobilize formerly unavailable soil components, including heavy metals, for uptake by their plant symbionts (Marschener 1998). AMF and other heavy-metal adapted fungi can immobilize HMs through the secretion of HM-binding glycoproteins such as glomalin (Gonzalez-Chavez *et al.* 2004) and through hyphal binding, with fungal hyphae having a 2-4 times higher affinity for HMs than plant roots do (Joner *et al.* 2000). Fungi that have adapted to survive in heavy metal contaminated conditions are likely the best candidates for remediation (Raman *et al*. 1993). In addition to their bioremediatory abilities, plants and soil fungi aid in the formation of soil aggregates (Gaur and Adholeya 2004), and help to prevent the erosion of contaminated tailings from the site and into nearby waterways. In order to better understand the bioremediatory abilities of HM-adapted fungi, we have set out to identify indigenous

fungi from Kam Kotia, an abandoned zinc and copper mine, and to culture these species *in vivo* and *in vitro*.

Contaminated soil from the Kam Kotia mine site and comparable uncontaminated soil (control treatment) was analyzed by the Queen's Analytical Services Unit using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES). The copper, zinc, arsenic and sulfur content of the soil was much higher in contaminated soil than in the control soil analyzed (see Figure 1C). Soil was also collected from the root systems of plants at the Kam Kotia mine in Timmins, ON for use in generating trap cultures to propagate environmental samples of fungi (see Figure 1A). Trap cultures are used to create a microcosm of the soil ecosystem in which environmental conditions are ideal for the growth, and later for the sporulation, of plant-associated fungi (see Figure 1B). Maize (*Zea mays*) was used as the host plant for our Kam Kotia trap cultures. Fungi were cultivated for 3 months with minimal watering to keep the maize alive, but stressed, so that fungal symbiosis would be encouraged. After 3 months of trap culture cultivation, root sections were harvested, DNA was extracted and the polymerase chain reaction (PCR) was performed using primers intended to detect AMF and the resulting fragments were subjected to DNA sequencing. The sequences were then aligned with the MaarjAM and NCBI genomics databases using a Standard Nucleotide BLAST search. Of the ten fragments successfully sequenced, nine were aligned most closely with the fungi *Capnobotryella sp. MA 3612* with a sequence identity of 85% for the Internal Transcribed Spacer region. One of the fragments sequenced aligned most closely with the fungi *Aureobasidium pullulans* with a sequence identity of 91% for the ITS region.

These fungi are known to associate with plants, but are not members of the division Glomeromycota and are not known to associate with plants in a obligately symbiotic fashion. Members of the genus *Capnobotryella* are black fungi that have been found to grow in association with the heavy metal hyperaccumulator *Thlaspi praecox* in heavy metal contaminated soil (Pongrac *et al.* 2009), suggesting their possible involvement in phytoremediatory processes. *Capnobotryella sp.* are thought to form ectomycorrhizae, but this genus and its associations with plants are not well understood. Further research must be carried out before *Capnobotryella* can be optimized for use as a bioremediatory organism. A lack of obligate symbiosis between *Capnobotryella* and plants could prove to be an advantage: if *Capnobotryella sp.* do indeed benefit associated plants in heavy metal contaminated soil, they may prove easier and more practical to culture *in vivo* and *in vitro* than AMF. *Aureobasidium pullulans* is a species of black fungi that is used as a biocontrol agent in agriculture and for the biological synthesis of polysaccharides and antifungal agents. This fungus is not an AMF and is not known to associate with plant roots. It is commonly found in extreme environments, and is able to survive drought and high levels of salt and heavy metal pollution (Gostinčar et al. 2014). Further chraracterization of the soil microbiome at Kam Kotia may allow us to determine whether *A. pullulans* is a primary succession species in the soil ecosystem of heavy metal contaminated sites, or if it is simply one of the only organisms equipped to survive in such an environment.

The sequences generated by this work are an interesting first look into the soil microbial communities of this heavy metal contaminated site. Future sequencing projects will allow us to better understand the diversity of the soil microbial community present at Kam Kotia. Because no AMF were isolated from the Kam Kotia site, a broader sampling approach is necessary to determine which plants at the site form mycorrhizal associations while experiencing heavy metal stress. The next step towards the optimization of these heavy metal tolerant fungi for use in bioremediation is the establishment of *in vivo* and *in vitro* fungal monocultures. *In vivo* monocultures of fungi will be established by inoculating soil with the desired fungal species and planting the seeds of a generalist host plant such as plantain (*Plantago major*) or maize. The creation of *in vitro* monocultures is a more challenging task, and requires the isolation of single viable spores. Spores will then be sterilized and planted alongside *Agrobacterium*-transformed hairy root cultures (see Figure 1D) of varying species to induce their germination, the formation of a symbiosis, and eventually the production of spores by the fungus. Our laboratory maintains a collection of 4 species of hairy root culture (chicory, tomato, carrot and potato). Fungi that form symbioses with plants have been found to exhibit preferences for certain hosts (Khan 2006). This diverse collection is expected to enhance the diversity of fungi that can be maintained in our *in vitro* cultures. These monocultures will be used to inoculate heavy metal tolerant plant species and investigate their impact on phytoremediatory processes. Propagules from these monocultures will also be used to determine how long AMF propagules persist in the soil record, whether they would be harmed by the application of fertilizers or chelating agents, and whether their tolerance for heavy metals is lost when grown on uncontaminated soil.

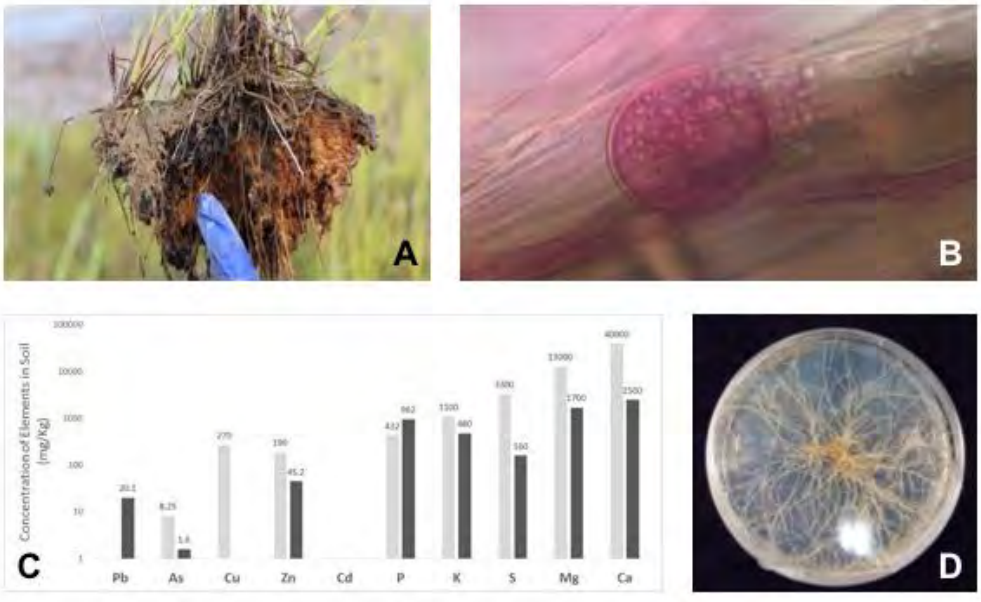

Figure 1. A) The root system of a grass growing in mine tailings at Kam Kotia. **B)** Fungal vesicle and hypha in plant root cortex. Fungal tissue stained with 0.04% fuchsin acid solution. **C)** ICP-AES analysis of soil from the Kam Kotia site. Concentrations in

parts per million (ppm). Light gray columns represent contaminated soil, dark gray columns represent control soil. **D)** *Agrobacterium*-transformed hairy root culture of chicory (*Cichorium intybus*) for use as a host plant in fungal *in vitro* cultures.

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Literature Cited

Gaur, A. and A. Adholeya. 2004. Prospects of arbuscular mycorrhizal fungi in phytoremediation of heavy metal contaminated soils. Current Science 86:528-534.

Giasson, P., A. Jaouich, P. Cayer, S. Gagné, P. Moutoglis and A. Massicotte. 2006. Enhanced phytoremediation: A study of mycorrhizoremediation of heavy metal–contaminated soil. Remediation Journal 17:97-110.

Gonzalez-Chavez, M. C., R. Carrillo-Gonzalez, S. F. Wright, and K. A. Nichols. 2004. The role of glomalin, a protein produced by arbuscular mycorrhizal fungi, in sequestering potentially toxic elements. Environmental Pollution 130:317-323.

Gostinčr, C., R. A. Ohm, T. Kogej, S. Sonjak, M. Turk, J. Zajc, P. Zalar, M. Grube, H. Sun, J. Han and A. Sharma. 2014. Genome sequencing of four *Aureobasidium pullulans* varieties: biotechnological potential, stress tolerance, and description of new species. BMC Genomics 15:1.

Hetrick, B. D., J. F. Leslie, G. T. Wilson and D. G. Kitt. 1988. Physical and topological assessment of effects of a vesicular–arbuscular mycorrhizal fungus on root architecture of big bluestem. New Phytologist 110:85-96.

Jankaite, A., and S. Vasarevičus. 2005. Remediation technologies for soils contaminated with heavy metals. Journal of Environmental Engineering and Landscape Management 13:109-113.

Joner, E.J., R. Briones, C. Leyval. 2000. Metal-binding capacity of arbuscular mycorrhizal mycelium. Plant Soil 226:227–234.

Khan, A. G. 2006. Mycorrhizoremediation - an enhanced form of phytoremediation. Journal of Zhejiang University Science 7:503-514.

Marschener, H. 1998. Role of root growth, arbuscular mycorrhiza, and root exudates for the efficiency in nutrient acquisition. Field Crops Research 56:203-207.

Mulligan, C., R. Yong, and B. Gibbs. 2001. Remediation technologies for metal-contaminated soils and groundwater: an evaluation. Engineering Geology, 60:193-207.

Pongrac, P., S. Sonjak, K. Vogel-Mikuš, P. Kump, M. Nečemer and M. Regvar. 2009. Roots of metal hyperaccumulating population of *Thlaspi praecox* (Brassicaceae) harbour arbuscular mycorrhizal and other fungi under experimental conditions. International Journal of Phytoremediation 11:347-359.

Raman, N., N. Nagarajan, S. Gopinathan and K. Sambandan. 1993. Mycorrhizal status of plant species colonizing a magnesite mine spoil in India. Biology and Fertility of Soils 16:76-78.

Schüßler, A., D. Schwarzott and C. Walker. 2001. A new fungal phylum, the Glomeromycota: phylogeny and evolution. Mycological Research 105:1413-1421.

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