Plant growth promotion on and phytoremediation of Athabasca oil sands coarse tailings using the endophytic fungus, *Trichoderma harzianum* TSTh20-1

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ABSTRACT

The environmental impact of bitumen mining in the Athabasca region of Canada is of growing concern. Among these concerns is the need and difficulty to remediate and reclaim affected land, including tailing sands (TS), a byproduct of the hot water extraction used to separate bitumen from solid materials. Current reclamation methods consist of multiple steps and take several decades to be effective. The primary reason for the difficulty in reclaiming disturbed land is the harsh environment found within the TS combined with the scale of the problem. TS are extremely nutrient poor, having below-detectable levels of NPK and extremely low C and S. In addition to this TS have pHs outside of environmental normals, and are hydrophobic due to residual hydrocarbons. Previously, an endophytic fungus, *Trichoderma harzianum* strain TSTh20-1, was isolated from pioneer plants growing naturally on TS sites, and was found to promote plant growth on TS. In my study TSTh20-1 was also found to increase the rate of drought recovery, and to enhance seed germination rates on a variety of soils. Suitable application methods were explored for this endophyte, including seed coatings, granules, as well as direct application to plant/soil. Regardless of method, TSTh20-1 was found to successfully colonize the plants.

Twenty-four species of grasses, forbs, and legumes were tested for their ability to grow on TS. The four most successful species (*Trifolium repens*, *Bouteloua gracilis*, *Medicago sativa*, and *Elymus trachycaulus*) were put into a seed mixture for use in experiments. In mesocosm-scale experiments, plant health and soil parameters were measured after 2 months of growth. Hydrocarbon analysis of the first mesocosm showed a 2.7-fold increase in total hydrocarbons when TSTh20-1 and plants were present, suggesting degradation of large hydrocarbons beyond the scope of the analysis. A repeat experiment using a different source of tailings did not yield this same result. This is most likely due using a source of tailings that had substantially different chemical characteristics. TSTh20-1 was also analyzed for its ability to produce plant hormones or siderophores, to increase peroxidase enzyme activity, to protect plants from reactive oxygen species, and to solubilize phosphate precipitates from soil. All of these are known mechanisms microbes use to promote plant growth.

ACKNOWLEDGMENTS

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Finally, I would like to thank the countless others whom I do not have space to thank individually.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Al	Aluminum
AMF	Arbuscular mycorrhizal fungi
B	Boron
C	Carbon
Ca	Calcium
CAS	Chrome-azurol S medium
Cu	Copper
DE	Diatomaceous earth
DNA	Deoxyribonucleic acid
Fe	Iron
$FeCl_3$	Iron(III) chloride
GA	Gibberellic acid
HTAB	Hexadecyltrimethylammonium bromide
IAA	Indole acetic acid
K	Potassium
KCl	Potassium chloride
KH_2PO_4	Potassium phosphate, mono-basic
Mg	Magnesium
$MgSO_4$	Magnesium sulfate
Mn	Manganese
Mo	Molybdenum
N	Nitrogen
NA	Naphthenic acid
NaCl	Sodium chloride
NH_4	Ammonium
NH_4Cl	Ammonium chloride
$(NH_4)_2SO_4$	Ammonium sulfate
NO_3	Nitrate

NPKS	Nitrogen, phosphate, potassium, sulfur
OA	Organic acid
OD	Optical density
PAH	Polyaromatic hydrocarbons
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PGP	Plant growth promotion
PIPES	Piperazine-N,N′-bis(2-ethanesulfonic acid)
PO_4	Phosphate
ppm	Parts per million
PSOL	Phosphate solubilization
PVAG	Polyvinyl alcohol glycerol
PVC	Polyvinyl chloride
PVK	Pikovskaya's medium
ROS	Reactive oxygen species
rpm	Rotations per minute
S	Sulfur
SO_4	Sulfate
TPH	Total petroleum hydrocarbons
TSTh20-1	*Trichoderma harzianum*, strain TSTh20-1
YE	Yeast extract
Zn	Zinc

LIST OF FIGURES

LIST OF TABLES

1.0. INTRODUCTION

The research presented in this thesis will characterize aspects of plant growth promotion and oil sand tailings bioremediation using a strain of the fungus *Trichoderma harzianum*, TSTh20-1. This is an endophytic plant symbiont that was isolated from a pioneer plant that naturally colonized a tailing sands (TS) site (Bao, 2009). TSTh20-1 has good potential for use in revegetation of TS and other dry and nutrient-limited soils. Specific study objectives are listed in section 1.5.

1.1. Endophytic Fungi

1.1.1 Endophytic fungi classifications and host ranges

The vast majority of septate fungal endophytes belong to the phylum Deuteromycetes, subphylum Ascomycota, with few belonging to subphylum Basidomycota (Rodriguez *et al*, 2008). Fungal endophytes can be divided into 4 classes depending on their function, transmission, host range, and colonization patterns, see Table 1.1.1 (Rodriguez *et al*, 2008). Class 1 endophytes are a small group of free-living and symbiotic species, mostly in the Clavicipitales (Redman *et al*, 2009). Their colonization is limited to few cool season grass hosts, and they are typically transmitted through the seed (Redman *et al*, 2009). Class 1 endophytes are defensive symbionts that are best known for producing toxins that make the host plant unpalatable to grazers (Bacon *et al*, 1977). The remaining three classes of endophytes include numerous fungal species that can colonize a wide range of plant hosts, both monocots and dicots (Redman *et al*, 2009). The fungus examined in this study, *Trichoderma harzianum* TSTh20-1, is a class 2 Ascomycete endophyte (Bao, 2009). Class 2 endophytes possess the ability to colonize all parts of the plant and form extensive networks of hyphae within the plant tissue. Class 3 endophytes form highly localized colonies within above-ground tissue, and conversely class 4 endophytes are limited to infecting below-ground tissue (Rodriguez *et al*, 2008). Class 2 endophytes have been shown to confer habitat-specific stress tolerance to a wide variety of abiotic stresses including salt, heat, nutrient, water, and metal stress. Class 2 endophytes can confer these tolerances to a wide range of hosts and demonstrate high colonization rates in plants growing on stressful environments (Redman *et al* 1999, 2001, 2002, 2008, 2011; Rodriguez *et al*, 2007). Class 2 fungal endophytes may also provide general benefits to the plant in a manner

similar to plant growth promoting bacteria, such as hormone production and enhanced nutrient uptake.

Table 1.1.1. Criteria used to classify fungal endophytic classes. Adapted from Rodriguez *et al* (2009).

Criteria	Class 1	Class 2	Class 3	Class 4
Host range	Narrow	Broad	Broad	Broad
Tissue(s) colonized	Shoot and rhizome	Shoot, root, and rhizome	Shoot	Root
***In planta* colonization**	Extensive	Extensive	Limited	Extensive
***In planta* biodiversity**	Low	Low	High	Unknown
Transmission	Vertical and horizontal	Vertical and horizontal	Horizontal	Horizontal
Fitness benefits	NHA	NHA and HA	NHA	NHA

1.1.2 Arbuscular Mycorrhizal Fungi (AMF)

AMF are affiliated with over 80 % of all terrestrial plants, and they are obligate biotrophs (Smith and Read, 1997). It is important to note that AMF do not fall within the classification of fungal endophytes provided in the previous section. They are a separate and distinctive class of organisms. The relationship between AMF and the host plant is mutualistic. The mycobiont absorbs mineral nutrients from the soil such as N, P, K, S, as well as micronutrients (Garg *et al*, 2006). It is believed that they provide these essential nutrients to the plant in exchange for fermentable carbon (Vierheilig *et al*, 1998; Smith and Read, 1997; Akiyama *et al*, 2005). Colonization of the root system by AMF allows the effective expansion of the rhizoplane to beyond what the roots themselves could achieve, leading to enhanced nutrient and water uptake (Kraus *et al*, 1987; Tawaraya *et al*, 2006). Bao (2009) studied the abundance of AMF present in plants (*Taraxacum officinale*) growing on TS. No difference in the colonization rates or abundance of AMF were found between unimpacted and TS sites. Rates of AMF colonization between sites was found to be abundant (~75 %) (Bao, 2009).

1.2. Class 2 Fungal Endophytes

1.2.1. Development of class 2 endophyte symbiosis

Similar to other endophytes or plant pathogens, class 2 endophytes penetrate plant tissue using hyphae or by means of specialized infection structures, such as haustoria (Ernst, 2003). Once within the plant tissue, the growth occurs primarily in intercellular spaces with limited damage to host cells (Rodriguez, 2008). Sporulation does not occur during active symbiosis, however it rapidly occurs through emergence during host senescence (Weber, 2004). The endophytes do not place any observable stress onto the host plant (Rodriguez *et al*, 2008). However, the maintenance of colonization of plants by class 2 endophytes appears to be partially dependant on stress being present. In a greenhouse study by Redman *et al* (2011) it was observed that only 65 % of plants were colonized under stress free conditions, whereas under stress colonization remained at 100 % throughout the 2 month duration of the study.

1.2.2. Culture of class 2 endophytes

Class 2 endophytes, unlike AMF (Tawaraya *et al*, 2006), can easily be cultured from sterilized plant parts on growth medium such as potato dextrose agar (Rodriguez *et al*, 2008; Bao, 2009). As a result they can easily be isolated from plants and subsequently purified. Since class 2 endophytes generally colonize the entire plant, they can be isolated from any part of the plant that has been surface sterilized. To-date no survey of fungal endophytes has been large enough to determine if Class 2 endophytes have a preferred location within the rhizosphere or whether they consistently extend beyond the rhizosphere (Rodriguez *et al*, 2009). The abundance of Class 2 fungal endophytes within the plant and rhizosphere appears to depend on species and likely varies at the strain level (Rodriguez *et al*, 2008). By this, it is best practice to always isolate Class 2 fungal endophytes from plant tissue rather than from rhizosphere soil.

1.2.3. Habitat-adapted symbiosis

Class 2 fungal endophytes have been shown to confer a wide range of stress tolerances (Rodriguez *et al*, 2008; Redman *et al*, 2011). The particular stress tolerances conferred by these endophytes is highly dependent on the environment from which they were isolated (Rodriguez *et*

al, 2008). For example, an endophyte isolated from a geothermal environment confers tolerance to heat stress but not tolerance to salt stress. Conversely, an endophyte isolated from a saline environment confers tolerance to salt stress but not heat stress (Rodriguez *et al*, 2008). In this study it was found that an endophyte isolated from a geothermal environment could not survive independently in elevated temperatures, nor could the plant. However, the endophyte and plant pairing could survive at elevated temperatures (Redman *et al*, 2008). Naturally occurring class 2 fungal endophytes have been isolated from a wide variety of high stress environments, and have been able to confer tolerance to each of these environments (Rodriguez *et al*, 2008; Bao, 2009; Redman *et al*, 2011)

Class 2 fungal endophytes can have dramatic effects on plant in both stress and non-stress conditions. Effects on host growth include, but are not limited to, increased biomass, reduced water use, increased reproductive yield, and greater resistance to pathogens (Bailey *et al*, 2006; Bao, 2009; Redman *et al*, 2011).

1.2.4. Potential mechanisms of host benefits

The mechanism(s) by which fungal endophytes confer habitat-specific stress tolerance has not been elucidated. It is likely that the method of stress mitigation is dependent on the type of stress. In their study of an endophyte that conferred thermal tolerance to plants, Marquez *et al* (2007) observed that the particular fungal endophyte contained a viral partner. When the viral partner was removed from the endophyte by freezing, the fungus lost its ability to confer heat tolerance to the plant. When the virus was reintroduced the fungus could once again confer this thermal tolerance to the host plant (Marquez *et al*, 2007). To further characterize this tripartite symbiosis, two RNA segments were isolated from the virus. The first of which was found to be responsible for virus replication within the host and the second has no known function or match with any known protein and remains unidentified (Marquez *et al*, 2007; Rodriguez, personal communication). This complex three-way symbiosis may not be representative of all fungal endophytes, but does suggest a molecular component to the symbiosis between the endophyte and host in the form of gene regulation.

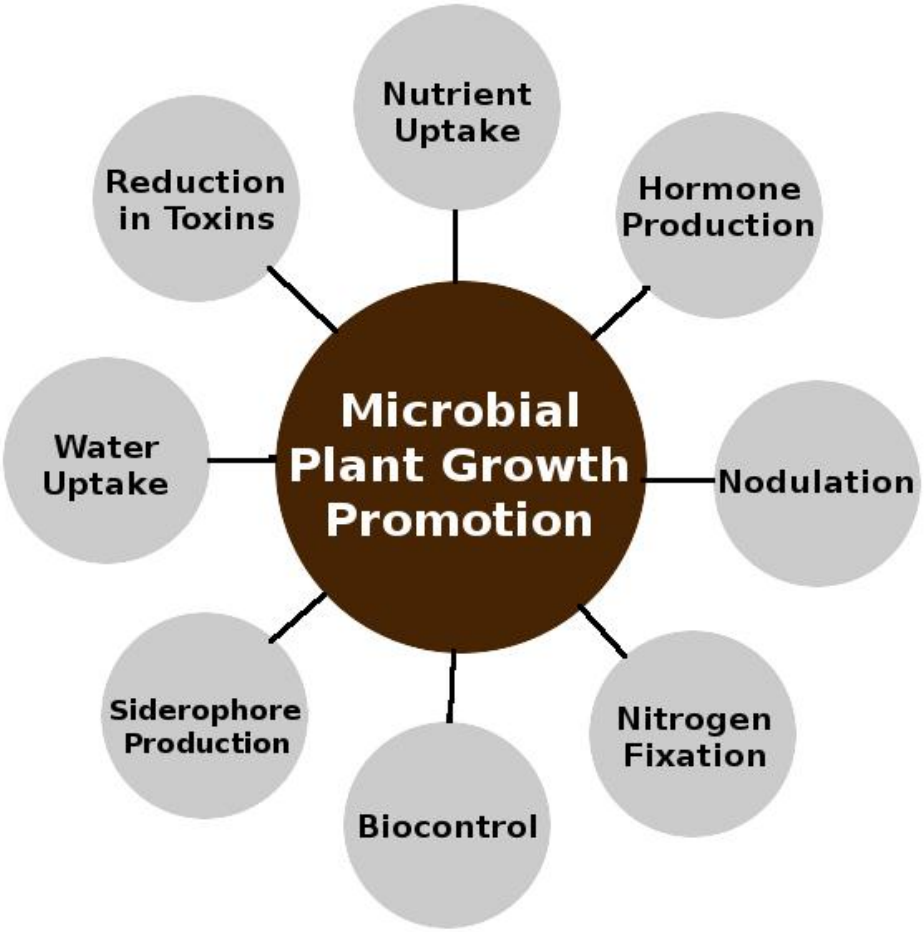

Figure 1.2.4. An outline of the basic methods used by bacteria and fungi to promote plant growth.

Alteration of gene expression levels of the host by endophytic *Trichoderma* spp. has been demonstrated in other studies (Bailey *et al*, 2006), where the gene expression patterns of the host and endophyte were observed to change during colonization. Bailey *et al* (2006) observed that gene expression patterns in both the endophyte and host change during infection, consistent with metabolic, regulatory, and signaling genes being regulated in both symbiotic partners. They also noted that the profile of expression changes was dependent on the endophyte strain used (Bailey *et al*, 2006). This suggests a complex genomic-level interaction between the endophyte and host. The fact that this interaction depends on the strain of endophyte used further suggests that the stress tolerances conferred by the endophytic partner will be specific to the endophyte and

environment from which it was isolated.

Chemical mechanisms of stress tolerance induced by fungal endophytes have not been studied in detail. However, these mechanisms have been studied in great depth in plant growth promoting bacteria (Hayat *et al*, 2010) and AMF (Artursson *et al*, 2006). Common mechanisms implicated in the promotion of plant growth by bacteria include alterations of mineral solubilization, siderophore production, and the production of hormones (Hayat *et al*, 2010). The solubilization of essential nutrients by both bacteria and fungi has been shown to increase plant biomass and apparent health (Guilden, 2000; Hayat *et al*, 2010). Due to microbial solubilization of key nutrients, most commonly phosphate, the plant can dedicate more energy to biomass production by not using energy reserves to solubilize nutrients from the rhizosphere. The production of siderophores by bacteria is believed to result in increased plant health (Hayat *et al*, 2010). Siderophores are molecules that chelate iron in solution, resulting in their increased solubility and bioavailability. Iron is an essential nutrient to the plant's energy generation processes, so by increasing iron availability the plant can increase its metabolic rate (Hayat *et al*, 2010). Finally, many plant growth promoting (PGP) bacteria and fungi have been shown to produce compounds similar to indole acetic acid (IAA), an auxin (Rodriguez *et al*, 2008; Hayat *et al*, 2010). This can have numerous effects on plant growth, but one effect of PGP fungi on plants that has been observed is the increase in both root hair abundance and length (Gulden *et al*, 2000). Increased root hair surface area can result in better water and nutrient uptake by the plant.

A review by Hamilton *et al* (2012) discusses the ability of endophytes to mediate the generation of reactive oxygen species (ROS) in plants. ROS play a role in programed cell death, metabolism, plant immunity, and stress response (Hamilton *et al*, 2012). Numerous studies outlined in that review demonstrate the ability of fungal endophytes to regulate ROS in plants, leading to reduced stress and resulting cell death. This effect has also been demonstrated with certain class 2 fungal endophytes (Rodriguez *et al*, 2004; Marquez *et al*, 1997). The ability of fungal endophytes to offset stress metabolites generated within the plant is likely part of the complex mechanism used in symbiosis to confer stress tolerance (Rodriguez *et al*, 2004).

Both fungal endophytes and plant pathogens have been found to release volatile compounds that can influence plant growth without physical interaction (Hung, 2013; Kang, 2013). The profile of volatile compounds released by the fungal partner is complex and can contain hundreds of compounds (Kang, 2013). Each of the volatiles produced by the fungus could have specific effects on the plant. Some volatiles have been shown to increase seed germination rates, whereas others result in increased root biomass in early stages of plant growth (Hung, 2013). The detailed roles of these volatile compounds are currently unknown, but they are believed improve host fitness before infection by the pathogen or endophyte (Kang, 2013). PGP bacteria have also been shown to exude volatile compounds that regulate both plants and fungi in the rhizosphere (Piechulla, 2013). These initial investigations of volatile compounds exuded from fungi and their effects on plants suggest that volatile metabolites could play a crucial role in plant-fungal interactions.

1.2.5. Hydrocarbon Degradation

Hydrocarbons present a challenge for microorganisms to degrade because they are hydrophobic and tend to tightly adhere to soil particles, resulting in low bioavailability (Covino *et al*, 2013). Degradation of straight-chain alkanes by microbes is typically by oxidizing one or both ends of the hydrocarbon chain (Callaghan *et al*, 2006). Once the alkane chain has been oxidized to a carboxylic acid, it can be degraded as normal fatty acids would be and used as an energy source by the microbes (Callaghan *et al*, 2006).

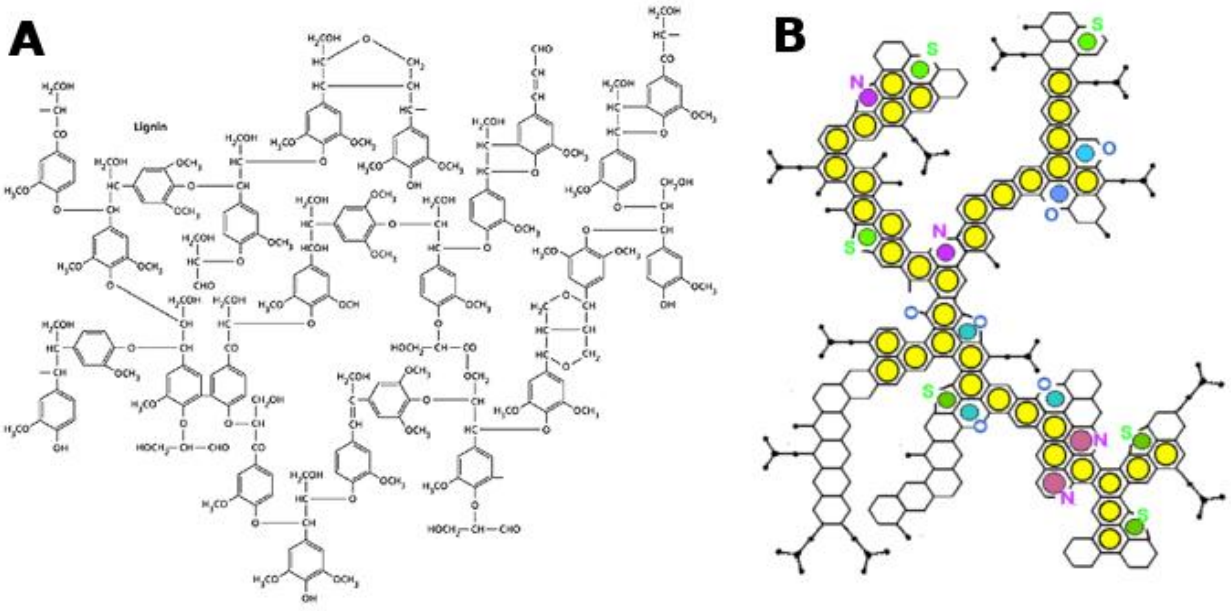

Figure 1.2.5. A comparison of the structures of lignin (A) and a sample asphaltene (B).

Polycyclic hydrcarbons are typically difficult for microorganisms to degrade. Their relatively large molecular size, hydrophobicity, and numerous types of bonds make them difficult for enzymes to access and act upon. However, some fungal enzymes are known to degrade large and complex molecules found naturally, including lignins (Covino *et al*, 2013). Enzymes produced by white-rot fungi, including manganese-peroxidase, lignin peroxidase, and laccase have been shown to degrade poly-aromatic hydrocarbons (PAHs) under *in vitro* conditions (Majcherczyk *et al*, 1998; Eibes *et al*, 2006). Recent studies have shown that under certain circumstances, the introduction of white-rot fungi to soils contaminated with PAHs results in a marked decrease in hydrocarbon levels. The ability of white-rot fungi and their affiliated enzymes to degrade hydrocarbons is likely due to the similarity in structures found between lignins and complex hydrocarbons (Figure 1.2.5). Oil sands are known to contain high levels of complex and polycyclic hydrocarbons known as asphaltenes (Ignasiak *et al*, 1979). Depending on the extraction conditions used by each company, these large PAHs may or may not be extracted with the bitumen fraction. Regardless of extraction method used, some hydrocarbons, including asphaltenes, are not extracted and end up in tailings.

1.3. *Trichoderma harzianum*

1.3.1. General information

Trichoderma harzianum is a species complex found over a wide geographic and environmental range. The numerous strains found within the species form a large complex with a range of phenotypic and genotypic variation (Chaverri *et al*, 2003).

Strains of *Trichoderma harzianum* are biocontrol agents used in commercial farming, as well as in the producers of cell well degrading enzymes (Naseby *et al*, 2000). These enzymes allow *T. harzianum* to degrade the cell walls of other fungi. The weakened cell wall is then penetrated, followed by a burst of antibiotics, killing the other fungus (Lorito *et al*, 1996). Once within the hypha, *T. harzianum* has been demonstrated to grow within the hyphal network of the other fungus, consuming cellular contents (Lorito *et al*, 1996). *Trichoderma harzianum* strains have also been found to alter the genetic expression of healthy plants, causing increased levels of defense genes and hormones, leading to increased resistance to a wide range of plant pathogens (Elad *et al*, 2000; Harman *et al*, 2004). Strain T-22 of *T. harzianum* has been demonstrated to solubilize key plant nutrients that are normally found as precipitates in the soil as well as leading to increase root growth when plants were grown in its presence (Altomare *et al*, 1999). In another study, *T. harzianum* T22 was shown to increase seed germination rates through the alleviation of biotic and abiotic stresses on seeds and seedlings (Mastouri *et al*, 2010). However, the mechanism of increased seed germination is not understood (Mastouri *et al*, 2010).

1.3.2. Strain TSTh20-1

In a previous study by Bao (2009), a strain of *T. harzianum*, TSTh20-1 was isolated from a dandelion (*Taraxacum officinale*) growing on a TS site in northern Alberta. This endophyte was found to significantly promote the growth of tomatoes on TS (Figure 1.4.1.1.). The *T. harzianum* TSTh20-1 endophyte strain was isolated by standard microbiological methods and identified using molecular and morphological means (Bao, 2009).

The mechanism(s) of PGP were not investigated in this previous study. However, TSTh20-1 was

examined for its ability to increase water use efficiency in plants grown on potting mix. This was measured by measuring the soil water content at the time of wilting. No significant differences in soil water content at the time of wilting were observed in this study (Bao, 2009). TSTh20-1 was also found to contain a plasmid as part of its genetic material. However, the role of this plasmid in symbiosis and its sequence remains undescribed (Redman, Personal Communication).

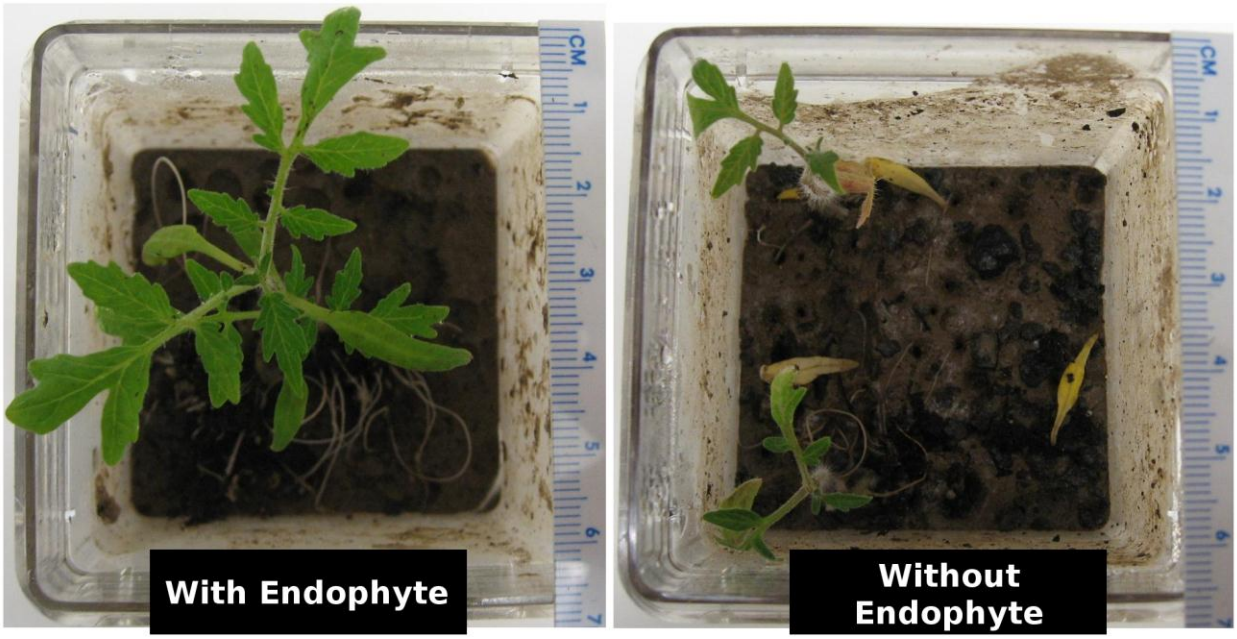

Figure 1.3.2. Fungal endophyte TSTh20-1 has been previously shown to enhance growth of tomato plants on TS (Bao, 2009).

1.4. Athabasca Oil Sands

1.4.1. Geographical and geological characteristics

The Athabasca oil sands in north-eastern Alberta (Figure 1.4.1.), Canada are the largest oil sands deposits in the world and are the world's third largest proven oil reserve (Royal Society of Canada Expert Panel, 2010; Radler *et al*, 2002). The Athabasca region is one of three oil sands mining areas in Alberta, and is by far the largest, accounting for over 95% of bitumen found in all of North America (Royal Society of Canada Expert Panel, 2010; Hein *et al*, 2000). The Athabasca oil sands have been known for over 200 years through the discovery of oil seeps along the Athabasca River, for which the region is named (Carrigy *et al*, 1963).

Figure 1.4.1. Map of North America, highlighting the oil sands deposits in Alberta. Three major oil sands deposits exist in Alberta: The Peace River, Cold Lake, and Athabasca deposits. The Athabasca deposit is the world's largest deposit of bitumen (Radler *et al*, 2002; Royal Society of Canada Expert Panel, 2010).

The Athabasca oil sands deposits are composed of 83-88 % inorganic materials, particularly sand and fine clay particles. These solids are mixed with 3-5 % water with the remainder being bitumen, a semisolid mixture of complex hydrocarbons derived from a variety of sources (Engelhardt *et al*, 2005; Budgell *et al*, 2006). The deposits of bitumen are found within a layer from the Lower Cretaceous period (Carrigy *et al*, 1963). There are several theories regarding their origin. The most prevalent theory suggests that they developed from Carboniferous shales 300-360 million years ago through coalification (Stanton *et al*, 2004). Coalification is the process of peat changing to coal through increased temperature and pressure.

As coal formation progresses there is a release of water and volatile organics (Stanton *et al*, 2004). In adjacent regions, over 650 billion tonnes of coal are known to exist, suggesting that the coal and bitumen arose from similar starting material and processes (Stanton *et al*, 2004).

1.4.2. Current and future scales of operation

Alberta's oil sands are one of the few hydrocarbon deposits, worldwide, that are experiencing growth in production. There are ~170 billion barrels of proven oil reserves and the total of all recoverable oil reserve is estimated at nearly 335 billion barrels (Alberta Government, 2011; AEUB, 2007; Chastko *et al*, 2004). Production may increase as new technology allows deeper bitumen deposits to be accessed. For surface mining to be economically viable, bitumen deposits must be no deeper than 70 meters below the surface. Deposits deeper than 70 meters can only be accessed using *in-situ* methods (Government of Alberta).

As of 2010, 767 km^2 have been disturbed by surface mining alone, and the area of disturbance could expand to 4800 km^2 using current technologies (Government of Alberta). Figure 1.4.2.1 illustrates the scale of current mining operations compared to a major U.S. city, Chicago. At 767 km^2 the area disturbed by surface mining operations are larger than the entire city, which covers 606 km^2. Figure 1.4.2.1 also shows the two key components of a bitumen mine. Surface mines are the location pits where mixtures of bitumen/sand mixtures are extracted from the ground. After processing, tailings are pumped as a slurry to tailings management areas where the solids are allowed to settle out of process water, which is then reused. Heavy particles, such as sand, rapidly settle forming coarse tailings; the remaining oil sands process water (OSPW) and fine tailings (FT) remain in tailings ponds until enough solids have settled out of the water for its reuse leaving FT behind.

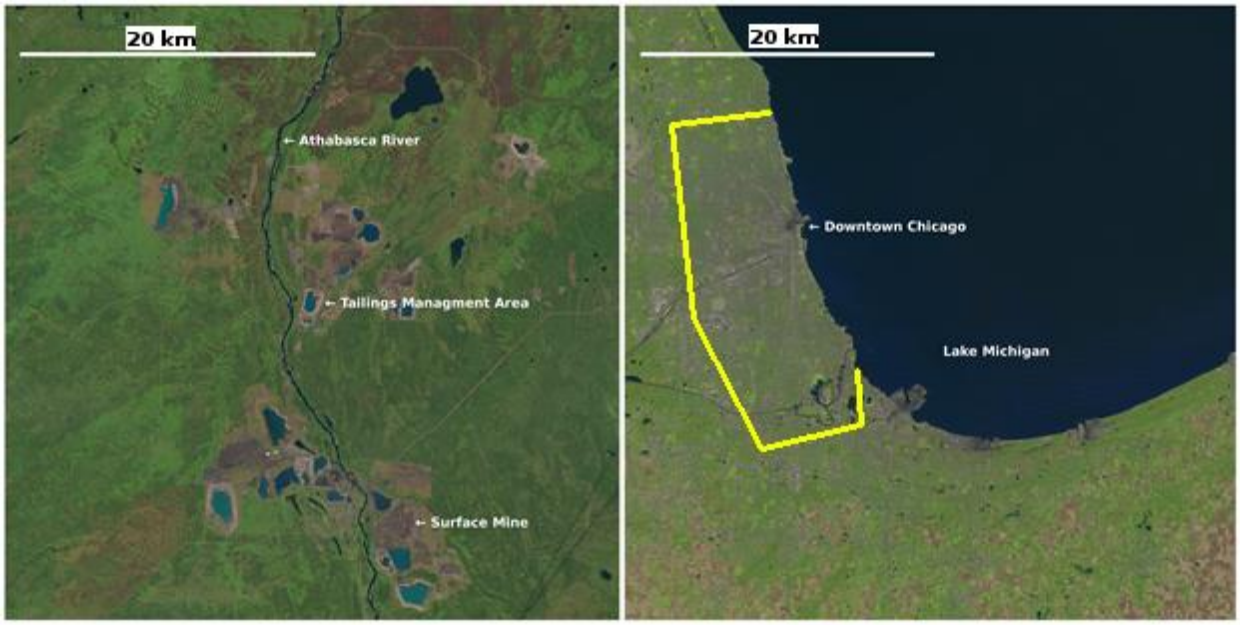

Figure 1.4.2.1. Satellite images showing the scale of oil sands mining operations compared to Chicago. The approximate outline of the Chicago city limits has been outlined in yellow. Landsat imagery courtesy of the United States Geological Survey (USGS).

An estimated 1.5 million barrels of oil is produced daily from the Athabasca oil sands region, a volume that is expected to increase to over 3 million barrels per day by 2018 (Government of Alberta). Estimates project that current and planned oil sands mining projects could increase production to as high as 6 million barrels per day by 2035 (International Energy Agency, 2012). As a result of mining expansion, the area disturbed by surface mining is expected to grow 6.7-fold to 4800 km^2. Legislation dictates that companies operating in the area must have a zero-discharge policy. This means that all tailings and process water must be held on site. OSPW is held in large tailings ponds, these ponds are projected to contain over one billion cubic meters (one trillion liters; one cubic kilometer) by the year 2025 (Han, 2008). These tailings ponds already cover an estimated 130 km^2 and will have to be augmented to meet the additional influx of OSPW (Government of Alberta, 2010).

Oil sands mining and exploitation have seen exponential growth over the past three

decades. This is very closely related to the surface area of land disturbed by mining operations as depicted in figure 1.4.2.2 (Statistics Canada). Current projections by the International Energy Agency expect this rate of growth, and thus land disturbance, to continue for several decades (International Energy Agency, 2012).

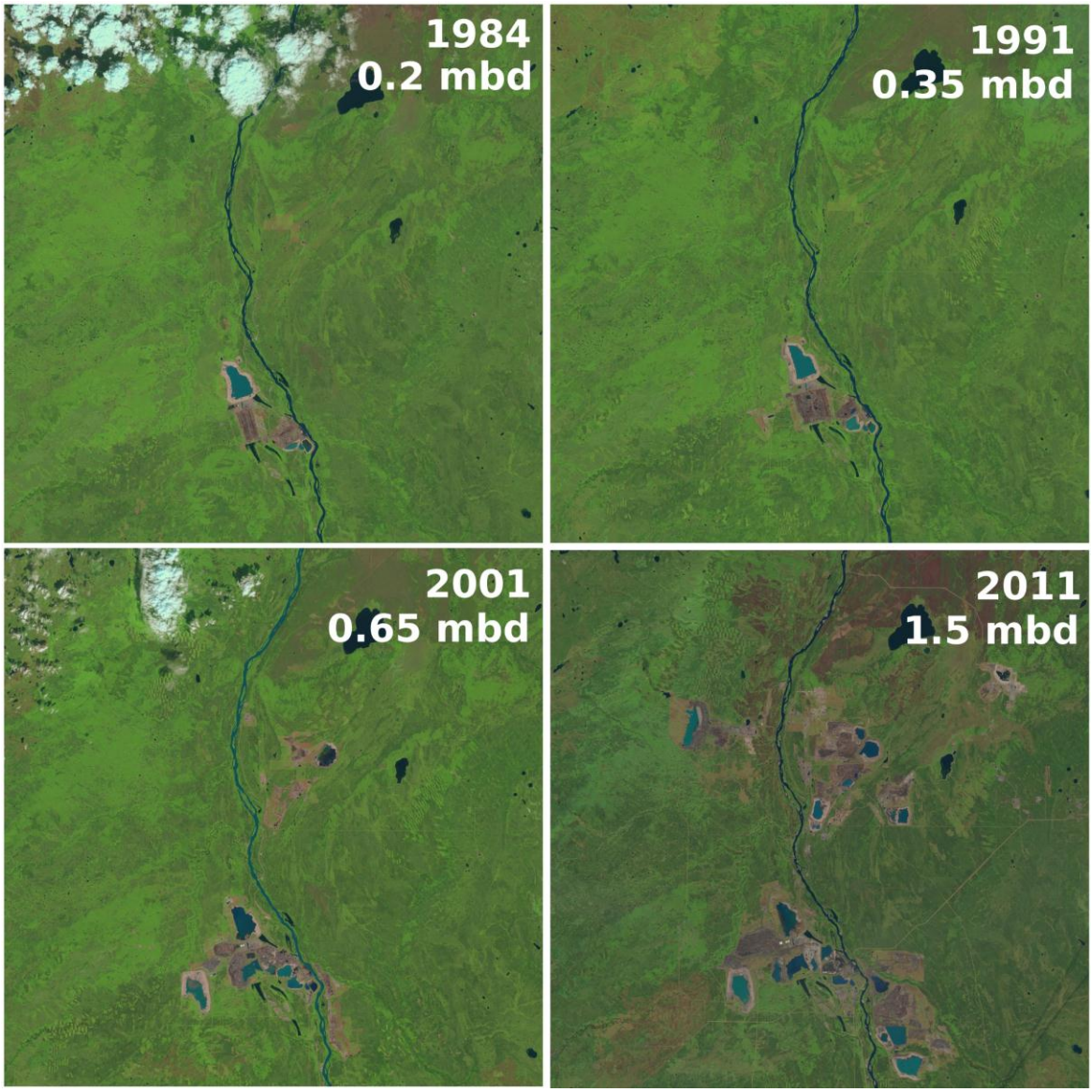

Figure 1.4.2.2. Satellite image showing growth of oil sands mining operations over the past three decades. Corresponding oil production in millions of barrels per day (mbd) data is from Statistics Canada). Landsat imagery courtesy of the United States Geological Survey (USGS).

1.4.3. Oil extraction process

Bitumen can be recovered by one of two processes, depending on the depth of the deposit. Deposits that are deeper than 70 meters are recovered using *in situ* methods. In general, this is done by pumping steam into the deposit. The added heat reduces viscosity the bitumen, which can then be pumped out of the ground and further refined (Bao, 2009). This method generates very few surface tailings because the extraction process specifically targets bitumen, leaving solids in place.

Surface mining is used for deposits that are less than 70 m deep. This process is outlined in Figure 1.4.3. Before mining can begin, the existing forest and organic soils are stripped from the land and stockpiled for reclamation purposes (Government of Alberta, 2010). The remaining overburden, or mineral soil, is removed and placed into tailings management areas (Government of Alberta, 2010). Once the bitumen deposit is exposed, it is mined using traditional methods including drag lines and transported to crushers. Crushers are used to make the raw bitumen an even size for mixing into a slurry. The slurry is composed of bitumen and sand mixed with water, and typically a dispersant such as NaOH (Albian Sands is the only company to use sodium citrate instead of NaOH) to bring the pH to near pH 8. The slurry is pumped to an extraction facility where bitumen is separated from OSPW and solids. The bitumen is sent for further refinement where it is treated with solvents to remove any remaining solids. The cleaned bitumen is then diluted with natural gas condensate before being sent to for refinement. The solids and water slurry is pumped to tailings management areas where the solids are allowed to settle from the water to become tailings. Two types of tailings are formed; coarse tailings composed of sand and gravel, and fine tailings composed of fine clay particles. Water is removed from tailings ponds and recycled (Patents: US 4240897 A, US 20130081981 A1, and US 5876592 A).

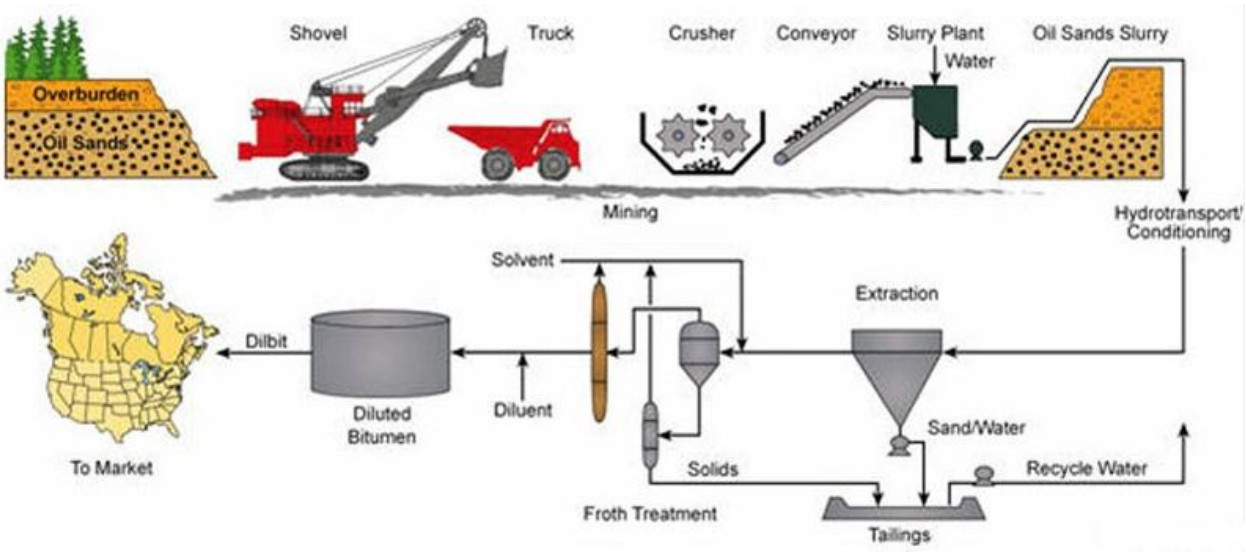

Figure 1.4.3. A generalized overview of surface mining techniques used to extract bitumen (Imperial Oil, 2012).

1.4.4. Legal requirements of reclamation and remediation

Before any oil sands mining project is permitted to begin, the company must submit reclamation plans for restoring the future affected area. The Environmental Protection and Enhancement Act (EPEA) calls for oil sands companies to "conserve and reclaim the disturbed land with the objective of returning the land to an equivalent land capability". Companies are also required to pay a bond to the Government of Alberta that will be used for reclamation if the company fails to properly reclaim on their own (Government of Alberta, 2010). If a company does successfully reclaim its disturbed lands, the bond paid to the government will be released back to the company (Government of Alberta, 2010). Upon completion of reclamation, the site is subject to 15+ years of monitoring to ensure that the restored landscape functions as a complete ecosystem (Government of Alberta, 2010). The complete 230+ pages of legal requirements, guidelines, and monitoring processes for reclamation can be found in: Guidelines for Reclamation to Forest Vegetation in the Athabasca Oil Sands Region, which is publicly available through the Government of Alberta (2009).

1.4.5. Current reclamation and remediation strategies

Once an area of land is no longer being used for active mining operations it is designated as "ready for reclamation." The first stage in reclamation is to fill the land, which is often the remnants of pit mines, with tailings and overburden. In the process the land is given a natural shape that should include habitat for wildlife such as embankments for burrowing animals. Once the land has been filled, it is covered with a 1 m thick layer of mixed peat and mineral soil that was stockpiled at the start of mining operations in the area. To stabilize this soil until further reclamation can occur, a mixture of grasses is planted. At this stage regular fertilizations are used to maintain healthy growth of plants. Over the course of several months to years, the grasses are replaced with a variety of natural species (as designated in the Guidelines for Reclamation) at natural densities. These natural species are maintained with fertilizer until they are able to grow without human intervention. At this point the land is designated as under "Permanent Reclamation". The 15+ year monitoring process begins to determine if the area has returned to a natural, stable, state per EPEA. (Government of Alberta, 2009). This process may take several decades depending on the site.

Due to their more complex ecology, there are currently no reclamation guidelines for restoring wetland sites: guidelines exist only for upland, dry, sites (Government of Alberta, 2009). Currently Syncrude, a major oil sands mining company, is undertaking an experimental project to restore a wetland site to a natural state (Syncrude, 2010). Restoration of wetland sites is important because of the large amount of area they cover in the Athabasca region. Wetlands are estimated to cover near 65 % of the surface area affected by mining operations (Foote, 2012). Current reclamation plans for wetland sites mostly involve a process known as water-capping, where a tailings pond is layered with clean water and left as a lake (Syncrude, 2010). This reclamation method results in the restoration of water rich areas, but does not restore the massive amount of peatland that was lost in the mining process, leaving the area in an unnatural state (Rooney *et al*, 2011). The loss of wetland has the potential to result in the release of over 45 million tonnes of carbon into the atmosphere and to reduce carbon sequestering capacity in the area by up to 7.4 tonnes of carbon dioxide per year (Rooney *et al*, 2011).

1.4.6. Projected costs of reclamation under current methods

Since only a small area, 1 km^2 (Government of Alberta) has been deemed under permanent reclamation the estimated costs of reclamation must be taken with some caution. However, reclamation costs of upland, dry, sites have been shown to range from $20,000 to $46,000 per hectare, and the projected cost of Syncrude's experimental wetland site is estimated to cost as much as $375,000 per hectare (Pembina Institute, 2010).

Because the reclamation of wetland sites is still in an experimental phase, it is likely costs will decrease as better methods are found. For simplicity, all disturbed areas will be treated as if they are upland sites. Assuming a median cost of $33,000 per hectare to restore upland sites, it would cost an estimated 2.4 billion dollars to reclaim the current 767 km^2 of disturbed land. If all 4800 km^2 of potential disturbance occurs this cost rises to 15.8 billion dollars for reclamation. The cost of wetland sites and tailings ponds will likely result in both of these estimates being much too low (Pembina Institute, 2010).

As discussed previously, current legislation requires the payment of a bond to the Government of Alberta, which will be used to pay for reclamation should the companies fail to do so. As of 2011, the government held 1 billion dollars in security bonds for this purpose (Government of Alberta, 2011). This is less than half of the dollar amount required to fully revegetate and restore the currently disturbed lands. This gap will likely grow as mining operations expand leaving the Canadian taxpayers at financial risk if companies fail to properly revegetate.

1.4.7. Previous studies of tailing sands (TS) soil environment

Very little research has been done to show the soil conditions found in coarse tailings. Previous work by Bao (2009) has shown TS generated by one company to be low in macro plant nutrients, low in organic carbon, hydrophobic, and with a moderately alkaline pH. No other studies of TS soil conditions have been located in the literature, nor have tailings generated by different extraction methods been compared. It appears that some of these data exist, but they are proprietary and not available even to the general scientific community. Without this information

it is difficult to generate standards and protocols for the revegetation of coarse tailings sites.

Limited studies on the environment presented by fine tailings (FT) and OSPW are publicly available. Wu *et al* (2011) studied the availability of key nutrients in FT and OSPW. The source of the OSPW and FT was not provided, therefore it is not known what extraction method was used to generate these tailings. These tailings were found to be low in key macro-nutrients such as nitrogen, phosphate, and potassium. However both OSPW and FT were high in sodium and chloride. pH was within half a unit of neutral in all samples measured (Wu *et al*, 2011).

No scientific data regarding the hydrocarbon content or distribution in OSPW, FT, or CT is publicly available to the best of our knowledge. However, the Alberta Energy Institute (2008) reports on the extraction methods, the percent of hydrocarbons recovered, and the treatment of large hydrocarbons such as asphaltenes by each major company operating in the Athabasca region. Both Syncrude and Suncor utilize NaOH in their slurry. This results in a >90 % extraction efficiency for both companies due to the greater amount of asphaltenes, complex polycyclic hydrocarbons, being extracted with the bitumen. By accepting these complex hydrocarbons into the refining process a large amount of coke, a coal like material, is generated during the upgrading process, which is classified as fuel, but generally is not sold due to pollution concerns. This method of extraction is most typically used by bitumen mining companies.

In contrast, Albian Sands uses sodium citrate to process their slurry (Devenny, 2009). Complex hydrocarbons such as asphaltenes are rejected from the Na citrate process and sent with tailings. This results in a lower overall extraction efficiency, but results in no coke being generated during the upgrading process. It also generates fewer fine tailings because the chemistry of the fine clay particles is not altered by the hydroxide ions. This difference in extraction methods likely has implications in the resulting chemistry of the coarse tailings. For example we would expect Albian Sands tailings to have a higher hydrocarbon percentage than Syncrude tailings due to the asphaltenes being rejected by Albian Sands (Devenny, 2009). This

difference in tailings chemistry may require different reclamation approaches to be used depending on the type of tailings on hand.

1.4.8. Naphthenic Acids

Naphthenic acids (NAs) are a complex group of organic acids. They typically contain either a 5 or 6 member cyclical structures attached to an alkane like structure (Rogers *et al*, 2002). Their mass can range from 100 to over 800 atomic mass units and can contain numerous carbon atoms (Rogers *et al*, 2002). NAs are naturally found in petroleum deposits and can be generated as part of the oil refinement process by oxidation of the naphtha fraction of crude oil. Typically, NAs are more of a corrosion concern than an environmental one.

However, NAs are one of the most researched contaminants produced by Athabasca oil sands production (Rogers *et al*, 2002). During the hot water extraction utilized in mining operations, NAs are liberated from the crude oil and dissolve into the water fraction as a sodium salt. Due to the solubility of NAs, they are widely regarded as an aquatic toxin (Nero *et al*, 2005). In toxicology studies, NAs have been demonstrated to be acutely toxic to fish (Nero *et al*, 2005). Similar studies have found that high doses of NAs can be acutely toxic to adult rats and chronic exposure at a lower dosage can result in liver and organ damage (Rogers *et al*, 2001). In a study on the effects of NAs on a deciduous tree species, it was found that NAs inhibit root water transport, leaf growth, and gas exchange (Kamaluddin *et al*, 2002). To date, no studies publically available have been performed assessing the quantity of NAs found in coarse tailings generated by oil sands mining.

1.5. Study objectives

The primary objective of this research was to better characterize *Trichoderma harzianum*, strain TSTh20-1, and determine if it can be used effectively to assist in reclamation and remediation of sites that have been affected by oil sands development. The secondary objective was to determine the most effective application methods and host plant species to use in potential future field studies.

1.5.1. What are suitable application methods for TSTh20-1 for plant colonization?

Previous studies of Class 2 fungal endophytes inoculated fungal spores directly to a young plant (Bao, 2009; Redman *et al*, 2011). This type of application is not suitable to field conditions because of the time-consuming nature and difficulty of planting germinated seeds. It was hypothesized that colonization would be possible using a variety of seed coating methods or soil application methods similar to commercial bacterial and fungal inoculations. Once an effective inoculation method is identified it can be used in future experiments using this endophyte.

1.5.2. Does TSTh20-1 confer abiotic stress tolerance related to growth on TS?

Tailing sands contain multiple stressors: they are nutrient poor, hydrophobic, and likely hydrocarbon-contaminated. Therefore, it is likely that TSTh20-1 is capable of conferring multiple stress tolerances. In this research TSTh20-1 was tested for its ability to confer drought tolerance, drought recovery, saline tolerance, as well as for its ability to liberate essential plant nutrients from soil. Other endophytic strains of *Trichoderma harzianum* have been shown to increase the germination rates of seeds; TSTh20-1 was tested for similar effects with and without stress present.

1.5.3. What native plant species demonstrate strong growth on tailing sands?

Twenty-four native and naturalized species of grasses and forbs were tested for their ability to confer strong growth on TS without the presence of TSTh20-1. It was hypothesized that plants able to grow well without TSTh20-1 would grow even better when the endophyte had colonized. Species were selected based on their native & naturalization status in Alberta (Government of Alberta; Alberta Reclamation Guide, 2004) as well as their cost when purchased commercially. Of the 24 species tested, 4 would be selected for a seed mixture that would be used in mesocosm scale experiments.

1.5.4. When paired with TSTh20-1, do native species demonstrate potential for remediation of TS?

Upon identification of suitable plant species and application methods, mesocosm scale

experiments were performed. In these experiments plant health parameters were measured including shoot length, root length, and biomass. Soil quality parameters including macronutrients (NPKS), soil organic matter, enzyme activity, and hydrocarbon levels were also measured. These were used as standards to determine the ability of TSTh20-1 to enhance reclamation as well as its ability to remediate the soil compared to control treatments.

1.5.5. Can any mechanisms of the conferred stress tolerance(s) be identified?

Preliminary work has been done to evaluate the potential mechanisms of stress tolerance provided by class 2 fungal endophytes. In this research TSTh20-1 was evaluated for its ability to confer tolerance to reactive oxygen species (ROS). It was also tested for its ability to produce extracellular organic acids and siderophores as well as solubilization of mineral nutrients. These mechanisms have been demonstrated either in other fungal-plant interactions as well as in plant growth promoting bacteria.

2.0. MATERIALS & METHODS

2.1. General procedures

2.1.1. Seed surface sterilization

Liquid sterilization

Tomato (*Solanum lycopersicum*, cv Rutgers) seeds were used for abiotic stress assays. Seeds were counted and placed into a steel mesh two-sided tea infuser for easy handling. The tea infuser was placed into 1 % (w/v) sodium hypochlorite (NaOCl) with moderate agitation for 15 min. After surface sterilization, seeds were rinsed using sterile ultrapure water (18 MegOhm from a Barnsted Nanodiamond) then placed in a sterile Petri plate for immediate use.

Gas sterilization

Small and sensitive seeds from native species were sterilized using chlorine gas. Seeds were counted and placed into a clean weigh boat so that there was no more than one seed layer thick. Fifty millilitres of household bleach (sodium hypochlorite, ~2.5 % w/v NaOCl) was placed into a 100 mL beaker or flask. Both the beaker of bleach and weigh boat of seeds were placed

into a large Tupperware® container. One milliliter of concentrated HCl was rapidly added to the beaker containing the bleach. The Tupperware container was quickly sealed and allowed to sit for 20-30 min. The chamber was opened in a fume hood, allowing chlorine gas to escape, seeds were transferred to a sterile Petri plate. Seeds could be stored like this for up to a wk before use without losing viability.

Verification of surface sterilization

To verify the surface sterility of seeds, a subset of seeds was plated onto 10 % PDA. Seeds were incubated for 1 wk allowing any colonies of bacteria or fungi to grow to a visible size. Plates were visually inspected for contaminants. For experiments where axenic conditions were essential, all seeds were plated on 10 % PDA and incubated until they germinated. Only seeds that had germinated and showed no signs of contamination were used for experiments. One plant species used in mesocosm scale experiments, Blue Grama (*Bouteloua gracilis*), contained an unidentified fungal endophyte [within the seed] that could not be removed by surface sterilization without killing the seed. This as yet unidentified endophyte grew from over 90 % of surface-sterilized seeds tested, but was not observed growing from surface-sterilized plant material where these seeds were used.

2.1.2. Growth media

Potato Dextrose Agar (PDA)

100 % PDA: 39 g of PDA powder in 1 L of ultrapure water, mixed until smooth. Medium was autoclaved using a 25 min 'liquid' cycle. After autoclaving, media was mixed and stored for future use. Alternatively, 100 % PDA was prepared by placing 24 g of potato dextrose broth (PDB) powder and 15 g of agar powder into 1 L of ultrapure water.

Ten percent PDA: 2.4 g of PDB powder and 15 g of agar powder were placed into 1 L of ultrapure water and mixed until no dry clumps remained. Media was autoclaved using a 25-minute liquid cycle. After autoclaving media was mixed and stored for future use. Alternatively, 10 % PDA was prepared by placing 3.9 g of PDA powder and 13.5 g of agar powder into 1 L of ultrapure water.

Chrome azurol S (CAS)

CAS media was prepared following the protocol developed by Alexander & Zuberer (1991) for the detection of siderophores. This method has been demonstrated to provide more consistent results than the original method provided by Schwyn and Neilands (1987). The media was prepared from 3 solutions: the indicator solution, the buffer solution, and the nutrient solution.

The indicator solution was prepared by mixing 10 mL of 1 mM $FeC1_3.6H_20$ in 10 mL of 10 mM HCl with 50 mL of 1.21 mg/mL aqueous solution of chrome azurol S (CAS). The resulting blue liquid was slowly added, with constant stirring, to a 40 mL solution of 1.82 mg/mL HTAB. The resulting dark blue solution was then autoclaved and then cooled to 50 ^{o}C.

The buffer solution was prepared by dissolving 30.24 g of PIPES in 750 mL of a salt solution containing 0.3 g KH_2PO_4, 0.5 g NaCl, and 1.0 g NH_4Cl. The pH was adjusted to 6.8 with 50 % KOH, and water was added to bring the volume to 800 mL. Fifteen grams of agar power were added, then the solution was autoclaved, then cooled to 50 ^{o}C. The nutrient solution contained 1 mL of a standard micronutrient solution and 2.4 g of PDB in 70 mL of water. The solution was autoclaved then cooled to 50 ^{o}C. The three solutions were mixed together with stirring, but avoiding introduction of air bubbles to the media. The media was poured into Petri plates for immediate use, but could be stored for several months at room temperature.

Pikovskaya's agar (PVK)

Pikovskaya's medium is used to detect phosphate-solubilizing microorganisms. It was prepared by mixing, per litre of water: 0.5 g yeast extract, 10 g glucose, 5 g calcium phosphate (hydroxyapatite), 0.5 g ammonium sulphate, 0.2 g potassium sulphate, 0.1 g magnesium sulphate, 1 mL micronutrient solution, and 15 g of agar. Media was autoclaved for 25 min, then allowed to cool to 50 ^{o}C. Cooled media was stirred to keep the hydroxyapatite in suspension then poured into sterile Petri plates. Pikovskaya's medium should be opaque, with hydroxyapatite spread evenly throughout (Sigma Aldrich).

2.1.3. Surface sterilization of plant material

Endophytes can be isolated from surface sterilized plant material. Since class 2 fungal endophytes are expected to colonize roots, shoots and leaves, either 1 cm root sections, entire leaves, or 5 mm punches of leaf material were sampled then surface sterilized. Plant material was placed into a steel tea steeper leaving ample room for the plant material to move within the steeper during agitation. The tea steeper was placed into a beaker containing 1 % (w/v) NaOCl for 5 min with moderate agitation. After surface sterilization, the plant material was rinsed using sterile ultrapure water, then placed into a sterile Petri plate for immediate use.

To verify surface sterility, the imprint method (Rodriguez *et al*, 2008) was used. Surface sterilized plant material was transferred to a plate of 10 % PDA. Plant material was pressed gently against the surface of the plate for 5-10 s, then was transferred from this plate. Imprinted plates were incubated for 1 wk. Plates were visually inspected for contaminants. If any were visible, surface sterilization had failed and the material was discarded.

2.1.4. Verification of axenic or inoculated conditions

Upon the completion of each experiment, plant material was sampled from both the control groups and experimental groups. The plant material was surface sterilized using previously described methods and plated onto 10 % PDA. Plates were incubated for 1 wk and visually inspected for fungal growth. Control plants were expected to have no fungal growth emanating from the plant tissues whereas the experimental groups should show fungal growth that matched the type of inoculant.

2.1.5. Dry silica stocks

Dry silica stocks were used for long-term storage of fungal spores. One and a half mL freezer tubes were filled to approximately the 1 mL mark with sterile anhydrous 200-mesh silica. Small (~5 mm) cubes of media were cut from a Petri plate that contained a sporulating fungal colony using a flame-sterilized scalpel. Three cubes of media were transferred to the freezer tube containing silica. Tubes were sealed and shaken vigorously for 5 min. After shaking the caps

were loosened from the tubes and they were placed in a desiccator for 24-48 h. Lids were then resealed and tubes were stored at either -20 ^{o}C or -80 ^{o}C. Samples were regrown from dry silica stocks by gently tapping a small amount of powder from the freezer tube onto a Petri plate containing 10 % PDA. Plates were incubated at room temperature until a mature sporulating colony was obtained.

2.1.6. Preparation of double-decker Magenta boxes

Double-decker Magenta boxes were prepared as per Redman *et al* (2010). For experiments where potting mix was needed, the top half of the double-decker Magenta box was filled halfway with potting mix (Sunshine Mix #1, Sun Gro Horticulture, USA) and the bottom half was filled with 250 mL of a 1 % fertilizer solution (Plant Prod All-Purpose 20-20-20 [NPK] water soluble fertilizer, Brampton, Ontario, www.plantprod.com). Prepared Magenta boxes were autoclaved for 25 min with the lids partially opened. After autoclaving, the lids were immediately sealed and the boxes were allowed to cool to room temperature. In experiments requiring the use of TS, the top half of the double-decker Magenta box was filled with 150 g of TS and the bottom half was filled with 250 mL of ultrapure water. Prepared Magenta boxes were autoclaved for 25 min with the lids partially opened. After autoclaving, the lids were immediately sealed and the boxes were allowed to cool to room temperature.

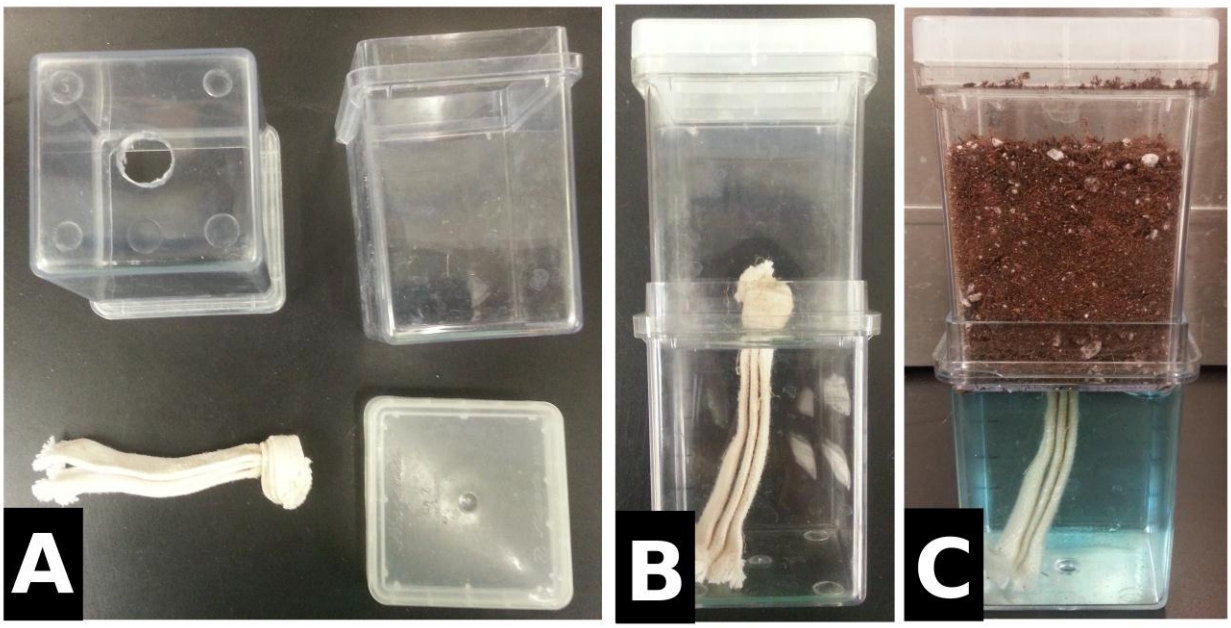

Figure 2.1.6. – Preparation of double-decker Magenta boxes. This image shows an example preparation of a double-decker Magenta box from its individual components (A) to assembled (B) to filled with soil and solutions (C).

2.2. Application methods

2.2.1. Liquid application to seedling

In previous endophyte studies (Bao, 2009; Redman *et al*, 2010), endophyte spores were applied directly to germinated seedlings. Seeds were surface sterilized and germinated on 10 % PDA as previously described. Spores of endophytic fungi were harvested in ultrapure water by rubbing the surface of a mature fungal colony with a sterile bent glass rod. The spore suspension was transferred to a 1.5 mL tube and diluted to 10^4-10^5 spores per mL as determined by hemocytometer. Seedlings were inoculated by transferring them to a Petri plate containing 20 mL of spore suspension. Seedlings were allowed to soak in this solution, with occasional agitation, for 30 minutes. After 30 min seeds were transferred to Magenta boxes for further germination and growth. Axenic controls were mock-inoculated by transferring them to a Petri plate containing sterile ultrapure water and allowing them to rest for 30 min, with occasional agitation. After the soaking, seedlings were transferred to Magenta boxes for further germination and growth.

2.2.2. Seed coating methods

Seed coatings are commonly used in commercial applications to carry germination enhancers as well as inoculants. Modern seed coating methods are complex and often contain several coatings combined together. To test the ability of TSTh20-1 to infect the plant from a seed coat, several rudimentary seed coating methods were devised: diatomaceous earth (DE) with a sucrose sticker, a polymer coating (polyvinyl alcohol with glycerol, PVAG), DE with a PVAG sticker, and dried alginate. Radish seeds were chosen for their large size and rapid germination. Fifteen coated seeds were planted in 500 mL pots filled with sterile potting mix, covered with 1 cm of vermiculite, and watered with ultrapure water. After 1 wk of growth plants were harvested, surface sterilized, and colonization assessed.

DE with Sucrose sticker

A number of seeds equal to 25 millilitres were treated in a sterile 50 mL conical tube with 1 mL of a 15% w/v sucrose solution that also contained 10^4-10^5 spores per mL as determined by hemocytometer. Seeds were shaken to ensure even coating with sucrose and spores. Seeds were then transfer to a fresh 50 mL tube where 1 g of dry DE was added. Seeds were shaken vigorously to ensure even coating with DE. Seeds were removed from the tube and allowed to dry for 24 h before use in experiments.

PVAG

Polyvinvl alcohol glycerol (PVAG) solution was prepared per Kaminskyj (2008) as modified from Brundrett *et al* (1996). The solution contains 4 g polyvinyl alcohol powder, 50 mL distilled water, 20 mL glycerol. This was warmed to 60 ºC, covered, with constant stirring until dissolved, typically 3 h to overnight. Five mL of room temperature PVAG were aliquoted and spore suspension was added to produce a spore density of 10^4-10^5 per mL. This was poured onto a 25 mL volume of seed in a 50 mL tube and shaken vigorously to ensure even coating. Seeds were removed from the tube and allowed to dry for 24 h.

DE with PVAG sticker

PVAG containing spores was prepared per the above method. This is poured onto 25 mL

of a seed in a 50 mL tube and shaken vigorously to ensure even coating. Coated seeds were transferred to a fresh 50 mL tube where 1 g of dry DE was added. Seeds were shaken vigorously to ensure even coating with DE. Seeds were removed from the tube and allowed to dry for 24 h.

Dried Alginate

A spore suspension was added to 30 mL of a 3 % w/v sodium alginate solution so that the final spore concentration was 10^4-10^5 spores per mL. Individual seeds were dipped into this solution then immediately dropped into a 5 % w/v calcium chloride solution with rapid stirring (Vipen *et al*, 2013). Seeds were gently removed and were placed evenly on trays in a 37 ^oC incubator for 24 h to dry.

2.2.3. Alginate beads

Alginate beads were prepared using a method similar to Vipen *et al* (2013). A spore suspension was added to 30 mL of a 3 % w/v sodium alginate solution so that the final spore concentration was 10^4-10^5 spores per mL. The spore alginate solution was dripped into a rapidly stirring 5% w/v calcium chloride solution causing the alginate solution to form gelatinous beads entrapping the spores. Freshly prepared alginate beads were used to inoculate plants immediately, but could be stored for 1 wk at 4 ^oC. Plants were inoculated by placing a bead next to each seed or seedling for slow growing endophytes. For rapidly growing endophytes, including TSTh20-1, placing 5-10 beads randomly placed in a Magenta box was adequate for inoculation of all plants in the box.

2.2.4. Charcoal pellets

Pellets were prepared by weighing 1 g of aquarium charcoal, approximately 300 pellets, (Fluval, www.hagen.com) and adding a spore suspension containing 50,000 total spores, as determined by hemocytometer, in a 50 mL conical tube. The mixture was shaken vigorously for 5 min and allowed to air dry at room temperature in the conical tube with the lid loose. The resulting pellets each contain approximately 170 spores. Spores on pellets prepared this way remained viable for several months (data not shown). Granules could be stored at room temperature for short-term storage or at -20 ^oC for long term storage. Plants can be inoculated by

placing a pellet next to each seed or seedling for slow growing endophytes. For more rapidly growing strains, such as TSTh20-1, placing 5-10 pellets anywhere in a Magenta box was adequate for inoculation of plants.

2.2.5. Direct application of spores to soil

The simplest and most rapid method to inoculate plants with TSTh20-1 was to apply spores directly to the soil. A sterile toothpick was pressed into a mature fungal colony so that the first 2-3 mm of the toothpick were covered with a visible layer of spores. The toothpick was then pressed into the soil near the centre of the Magenta box or pot at the same time the seeds or seedlings were planted.

2.3. Stress assays

2.3.1. Growth on tailing sands

To test the ability of TSTh20-1 to promote plant growth on TS double-decker Magenta boxes were prepared using previously described methods. Bao (2009) used seeds which had been germinated on Petri plates containing 10% PDA that had been inoculated with TSTh20-1 using liquid inoculation methods. After 2 wk of growth on potting mix, plants were gently transferred to Magenta boxes that contained TS. To decrease the number of steps and to make experiments more realistic, this study directly planted surface-sterilized seeds into Magenta boxes containing TS. Seeds were surface sterilized using previously described methods and 5 seeds were planted into each Magenta box. Boxes were inoculated with 5 charcoal pellets and sealed with their plastic lid to retain moisture during germination and early seedling growth. Double-decker Magenta boxes were grown in 16:8 h light:dark cycles for 2-4 wk at 21 ^{o}C. After the growth period plants were evaluated for their overall health using root wet/dry biomass, shoot wet/dry biomass, and root:shoot ratios.

2.3.2. Drought tolerance & recovery

Magenta boxes were prepared with potting mix using previously described methods. Tomato seeds were surface sterilized and 3 seeds were planted per Magenta box. Ten Magenta boxes per experimental group were used. Tomato plants were grown for 5 wk in 16:8 h

light:dark cycles at 21 ^{o}C refilling the bottom half of the Magenta boxes with sterile fertilizer solution as necessary. After 5 wk of growth, the bottom half of the Magenta boxes were emptied, removing the water supply from the plants. Plants were allowed to wilt for 36 h under the same lighting and temperature. One leaf was sampled from each Magenta box at 0, 18, and 36 h of wilting. Leaf samples were weighed fresh and then dried for 48 h at 55 ^{o}C, after which a dry weight was taken. After 36 h of wilt, the plants were rehydrated by pouring 200 mL of water over the potting mix and roots so that the water trickled into the bottom half of the Magenta box. One leaf was sampled from each Magenta box at 15, 30, 60, and 120 min after watering. Leaf samples were weighed fresh and then dried for 48 h at 55 ^{o}C, after which a dry weight was taken. Fresh and dry weights were used to calculate the weight percentage of water in each leaf. The Student's *t*-test was performed at each time point to determine significance ($P < 0.05$). The average water contents were graphed as a time course using 1 standard error for error bars and regression lines were assigned with their respective r^2 values.

2.3.3. Seed germination enhancement

White clover (*T. repens*) seeds were used to test seed germination enhancement since their small size makes them more susceptible to stress. Twenty 100 mL pots were filled with potting mix and another 20, 100 mL pots were filled with TS. Twenty-five white clover seeds were evenly spread over the soil surface of each pot. Ten pots containing potting mix and 10 pots containing TS were inoculated with 5 charcoal pellets containing TSTh20-1 spores. The other 20 pots were mock-inoculated with sterile charcoal pellets. Pots were incubated for 1 wk in 16:8 h light:dark cycles at 21 ^{o}C, and were watered with ultrapure water as needed. After 1 wk the number of germinated seeds was counted and the Student's *t*-test was performed to test whether there were significance in germination rates.

2.4. Selection of native species

2.4.1. Selection of candidate species

Twenty four native and naturalized species were chosen based on their preference for dry environments, coarse soils, native to Alberta, and commercially available through our chosen supplier; Prairie Moon Nursery (Prairie Moon Nursery, 32115 Prairie Lane, Winona, MN

55987, USA http://www.prairiemoon.com/). Seeds were divided into 2 groups; those requiring cold stratification and those that did not, roughly two groups of 12 species.

The forbes used in this study were: *Artemisia caudata* (Beach wormwood), *Aquilegia Canadensis* (Columbine), *Aster pilosus* (Frost aster), *Astragalus crassicarpus* (Ground plum), *Campanula rotundifolia* (Harebell), *Geum triflorum* (Prairie smoke), *Heuchera richardsonii* (Prairie alumroot), *Meticago sativa* (Alfalfa), *Penstemon calycosus* (Calico beardtongue), *Penstemon tubaeflorus* (Tube beardtongue), *Potentilla arguta* (Prairie cliquefoil), *Solidago nemoralis* (Oldfield goldenrod), *Trifolium repens* (White clover), and *Zigadenus elegans* (White Camass). The grasses used in this study were: *Agropyron smithii* (Western wheatgrass), *Agropyron trachycaulum* (Slender wheatgrass), *Andropogon scoparium* (Little bluestem), *Bouteloua gracilis* (Blue grama), *Calamagrotis canadensis* (Blue jointgrass), *Elymus Canadensis* (Canada wild rye), *Hordeum jubatum* (Squirrel-tail grass), *Juncus balticus* (Baltic Rush), *Poa palustris* (Fowl bluegrass), and *Sporobolus crytandrus* (Sand Dropseed).

2.4.2. Assay for growth on tailing sands

Two trays containing 50, 100 mL wells were surface sterilized with 70 % ethanol and allowed to air dry. Each well was filled with TS. Into one tray, the seeds requiring cold stratification were planted. Three to 10 seeds were planted per well depending on seed size: 3 large seeds or 10 small seeds with 3 replicate wells being planted for each species. Seeds were cold stratified as per germination instructions for 60 d at 4 ^{o}C (Prairie Moon Nursery, *personal communication*). Upon the completion on the cold stratification, the second tray was planted in a manner similar to the first with the seeds that required no stratification phase. Both trays were inoculated with TSTh20-1 and grown for 4 wk under 16:8 h light:dark cycles at a constant 21 ^{o}C and watered with a 1 % Tween 20 solution to reduce the hydrophobicity of the TS at the start of the experiment. After 4 wk of growth, seedlings were qualitatively evaluated for growth and health.

2.4.3. Seed mixture for use in mesocosm scale experiments

A seed mixture containing 4 species was developed using the qualitative growth data in the

selection experiment with the aims of creating a mixture that would support both short-term growth, long-term growth, and increased soil nutrient availably. Four candidate species were selected, and were planted together at 15 times their recommended seeding density indicated by the seed supplier in three 500 mL pots containing TS that had been treated with a 1 % Tween 20 solution to reduce hydrophobicity. All pots were inoculated with TSTh20-1 using 3-5 charcoal pellets and allowed to grow for 4 wk under 16:8 h light:dark cycles at a constant 21^{o}C, watering as necessary. At the end of 4 wk, pots were visually inspected for the presence of all 4 species and qualitative health. If all 4 species were present and relatively healthy the mixture was deemed suitable for meso-scale experiments.

2.4.4. Testing for competition effects

To ensure that one species would not dominate growth in meso-scale experiments the seed mixture was tested for competition effects. Thirty-two 100 mL test tubes were filled to 80 % capacity with TS that had been treated with a 1 % Tween 20 solution and arranged into 4 groups of 8 tubes. The first set of 4 tubes received 1 seed from each species, the second tube received 2 seeds from each species, and so-on until the final tube which received 8 seeds from each species. Seeds were covered with a 1 cm layer of potting mix and gently watered. Tubes were inoculated with TSTh20-1 using 3-5 charcoal pellets and incubated for 4 wk under 16:8 h light:dark cycles at a constant 21 ^{o}C, watering as necessary. After 4 wk each tube was evaluated for the presence of each species and qualitative health of plants in each tube (plant colour and height). If no one species was found to dominate growth, and all species were still present in significant numbers, and the plants appeared healthy, then competition effects were deemed negligible for future short-term experiments.

2.5. Mesocosm scale experiments

2.5.1. Version 1 – Tailing sands from company A

Tailings from Company A were used for the first mesocosm scale experiment. 12 PVC pipes 45 cm in length and 10 cm in diameter were cut lengthwise into 2 halves. These were held together using pipe clamps allowing the pipes to be easily opened and examined at the termination of the experiment. Eight pipes were filled with TS, leaving 5 cm clearance at the top

of the pipe. The remaining 8 pipes were filled with potting mix leaving 5 cm clearance. Each pipe was seeded with the seed mixture developed previously at 15 times the suggested rate to account for limited growth on TS.

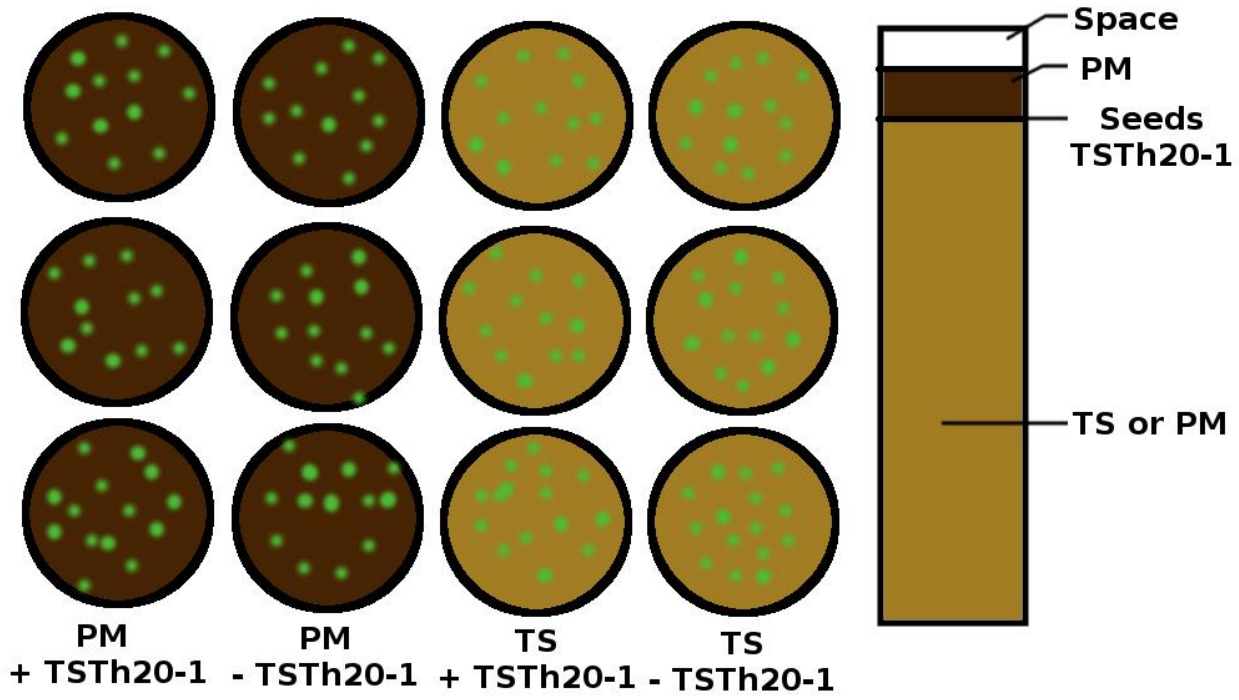

Figure 2.5.1.1. An outline of the design used in Version 1 (Company A) mesocosm scale experiment. The right portion of the figure shows how each tube was filled and the position of seeds within the tube.

Three pipes containing TS and 3 pipes containing potting mix were inoculated with 0.1 g of charcoal pellets containing TSTh20-1 (approx. 5000 spores) , the remaining 6 control pipes were inoculated with 0.1g of sterile charcoal pellets. Pipes were watered with a 1 % Tween 20 solution and grown in a phytotron chamber for 2 months under a 16:8 light dark cycle, 20^{o}C:11^{o}C day night temperature regime, and constant 80% relative humidity. These conditions are representative of Athabasca region in early summer (Environment Canada).

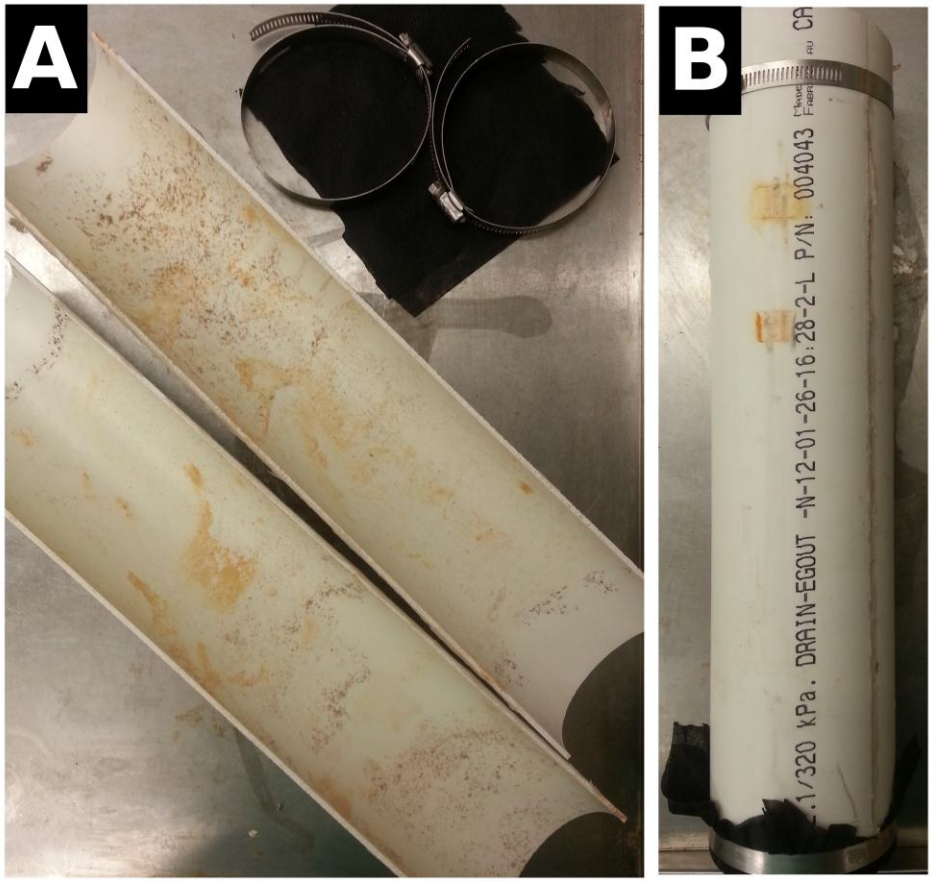

Figure 2.5.1.2. A brief outline of the assembly of the PVC pipes used in mesocosm scale experiments. (A) shows the necessary parts for assembling each tube; 2 halves of pipe, 2 pipe clamps, and 2 sheets of garden fabric. (B) shows the completed setup.

2.5.2. Version 2 – Tailing sands from company B

Due to limited quantities of tailings from Company A, tailings from Company B were used for a second mesocosm scale experiment. Thirty-six PVC pipes 20 cm in length and 10 cm in diameter and 5 PVC pipes 45 cm in length and 10 cm in diameter were cut lengthwise into two halves. These two halves were held together using pipe clamps allowing the pipes to be easily opened and examined at the termination of the experiment. Thirty pipes were filled with TS leaving 5 cm clearance at the top of the pipe. The remaining 5 pipes were filled with potting mix leaving 5 cm clearance. All but 10 pipes were seeded with the seed mixture developed previously at 30 times the suggested rate to account for limited growth on TS. The 5 pipes

containing potting mix were covered with 1 cm of potting mix and watered with a 1% Tween 20 solution. The 10 pipes that were not seeded were divided into 2 groups of 5. One group was inoculated with 0.1 g of charcoal pellets containing TSTh20-1 and the other was mock-inoculated with sterile pellets. The remaining 10 pipes were divided into 2 groups of 5. One group was inoculated with 0.1 g of charcoal pellets containing ~5000 spores TSTh20-1 and the other was mock-inoculated with sterile pellets. All pipes were covered with 1 cm of potting mix. Pipes were watered with a 1% Tween 20 solution at the start of the experiment, and water from thereafter and grown in a phytotron chamber for 2 months under a 16:8 h light dark cycle, 20 ^{o}C:11 ^{o}C day:night temperature regime, and constant 80 % relative humidity. These conditions are comparable to Athabasca region in early summer (Environment Canada). This experiment was designed to test the effects of TSTh20-1 on soil conditions with and without the presence of plants.

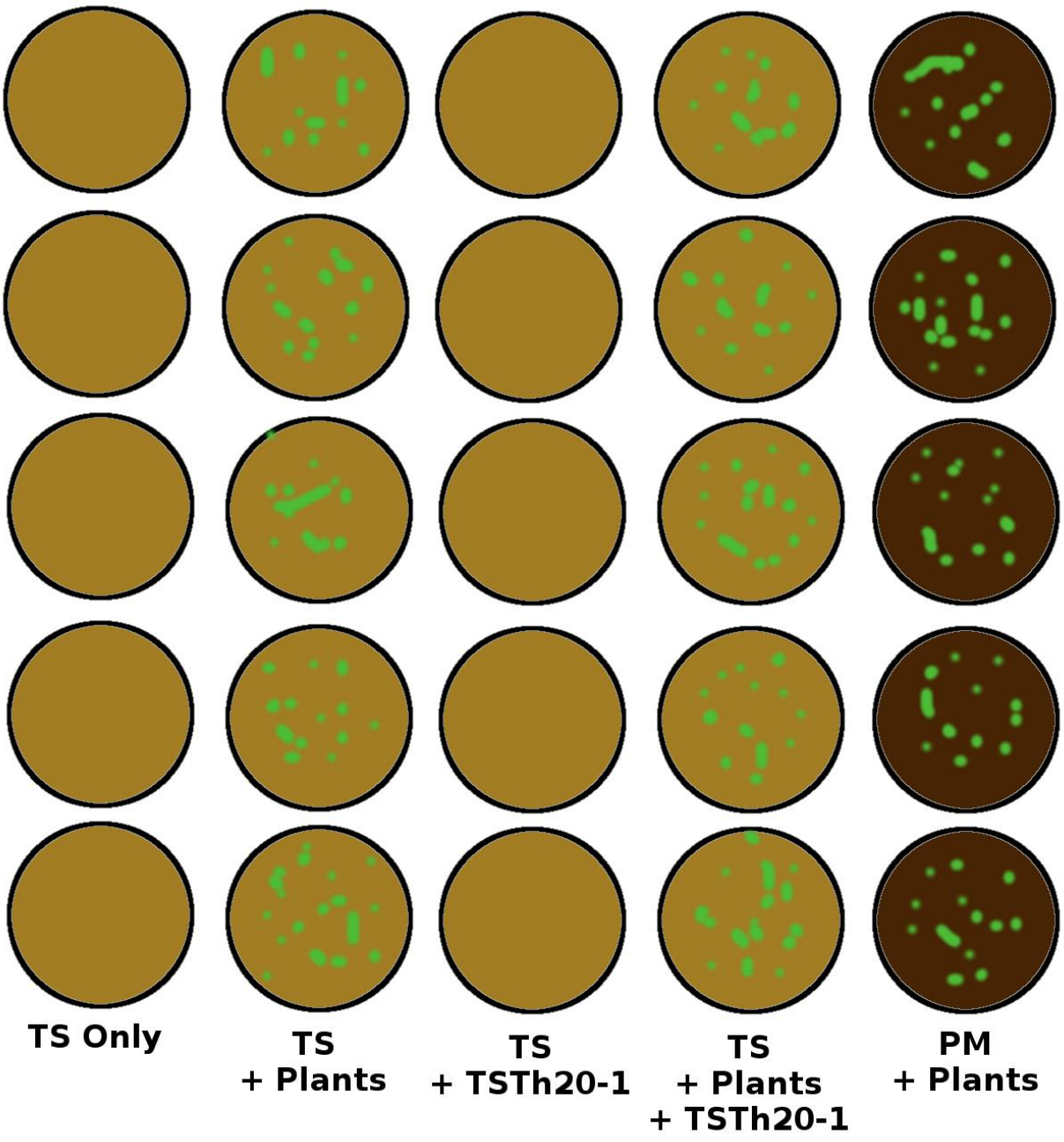

Figure 2.5.2.1. An outline of the experimental setup for the second mesocosm scale experiment (Company B).

Figure 2.5.2.2. PVC pipes in place in the phytotron chamber used for the mesocosm scale experiments.

2.5.3. Measuring plant parameters

In the first mesocosm scale experiment using tailings from Company A plant parameters were measured and statistically analyzed for significant differences in growth. At the completion of the experiment each pipe was opened lengthwise by removing the pipe clamps. From the pipes containing TS each individual plant was carefully removed from the pipe. For each plant species, pipe, treatment, root length, shoot length, wet root biomass, wet shoot biomass, dry root biomass, and dry shoot biomass were recorded. Dry weights were obtained by placing each plant in an individual weigh boat and drying them at 55 ^{o}C for 48 h. Plants in pipes containing potting mix were not analyzed, but root systems and leaf material was photographed.

2.5.4. Soil analysis

Company A

Soil samples were taken from within the rhizosphere from each tube containing TS and placed into 50 mL tubes. These tubes were evaluated for concentrations of potassium, sulfate, phosphate, nitrate, ammonia, and organic carbon. Equal portions of soil from each replicate pipe were sampled, mixed thoroughly, and placed into a 50 mL tube. These mixed samples were evaluated for hydrocarbons using the CCME guidelines (CCME, 2008). A sample of untreated tailings was taken and analyzed for nutrients and hydrocarbons as a baseline. All analyses were performed by ALS Environmental (http://www.alsglobal.com/).

Company B

Soil samples were taken from within the rhizosphere from each tube containing TS and placed into 50 mL tubes. All tubes were analyzed for hydrocarbons following the CCME guidelines (CCME, 2008). All analysis was performed by ALS Environmental (http://www.alsglobal.com/). The Student's *t*-test was performed on hydrocarbon levels to determine significant differences between treatments. Soil was also evaluated for cell-free peroxidase activity, see section 2.6.3 for method.

2.6. Mechanisms of plant growth promotion

2.6.1. Phosphate solubilization

Initial screening for phosphate solubilization (PSOL) was performed on plates containing Pikovskaya's media as prepared in section 2.1.2. Inconclusive results on this medium prompted a study of PSOL by TSTh20-1 in liquid cultures. Pikovskaya's medium was prepared without agar or hydroxyapatite to create a clear broth. Eighteen 50 mL flasks were filled with 25 mL of this clear broth. Flasks were divided into 3 groups of 6. Each group received equi-molar amounts of an insoluble phosphate precipitate commonly found in the soil: aluminum, calcium, and iron. Flasks were autoclaved for 25 min and cooled to room temperature. Each flask was inoculated with ~3000 spores, as counted by hemocytometer, of either *Penicillium bilaiae*, a known phosphate solubilizer (David Greenshields, *personal communication*), or TSTh20-1, and shaken for 1 wk at 150 r.p.m. At the start of the experiment and after 1 wk, 1 mL aliquots were sampled.

Each sample was filter sterilized and examined by HPLC for organic acid production. A 20 µL sample was then examined for the concentration of solubilized phosphate in the sample using the malachite green method (Feng *et al*, 2011).

2.6.2. Production of siderophores

TSTh20-1 was plated onto Petri plates containing CAS media and grown for 1 wk. This medium turns from blue to pink when siderophores are present. This test is universal and can detect all classes of siderophores (Perez-Midranda *et al*, 2007). Lack of colour change indicates that no significant amount of siderophores were detected, whereas a pink halo around the colony indicates that siderophores were being produced. *Penicillium bilaiae* was used a positive control to test the media since it is known to produce siderophores (David Greenshields, *personal communication*).

2.6.3. Soil enzyme activity

Soil cell-free peroxidase activity was tested following the procedure developed by Barth and Bordeleau (1969). Fifty grams of soil were suspended in 50 mL of 0.05 M phosphate buffer at pH 6.0. The suspension was agitated for 5 min then gravity filtered into a clean flask. The assay consisted of the following reagents mixed together into a 3 mL, 1 cm, cuvette: 0.05 mL 0.5 % *o*-dianisdine in methanol, 0.3 mL 0.06 % hydrogen peroxide in water, and 2.7 mL soil extract. After 2 minutes the optical density was recorded at 460 nm. Ultrapure water was used as a blank for the spectrophotometer and the negative control consisted of a cuvette where ultrapure water was used in place of the soil extract. Activity was calculated as the change in absorbance per min at 460 nm.

2.6.4. Tolerance to reactive oxygen species

Rodriguez *et al* (2008) reported an assay for testing the ability of fungal endophytes to protect against reactive oxygen species (ROS) in green (photosynthesizing) plant tissue. One millilitre of 10 mM paraquat solution, a herbicide that generates ROS in stressed plants, was pipetted into a 1.5 mL centrifuge tube. Leaf disks were taken using a hole-punch from 2 wk old tomato plants that had been grown on TS, creating leaf disks consistent in size. Leaf disks were

taken from both plants that had been inoculated with TSTh20-1 and axenic plants. The disks were gently placed on the surface of the paraquat solution, with each disk receiving its own tube. Disks were placed into a rack and left under bright fluorescent lighting for 24 h. After 24 h the amount of bleaching was evaluated using the scale provided by Redman *et al* (2008).

2.6.5. Hormone production

Hormone production was evaluated using a modified Ehrlich reaction. The reagent was prepared by mixing 2 g of *p*-dimethylamino benzaldehyde into 50 mL of 100 % ethanol. Then, 50 mL of concentrated HCl was added slowly, with mixing. The reagent was best used when freshly made (Hvorost *et al*, 2010).

A solid, nutrient-free growth medium was prepared by making 10 % PDA and adding an addition 1 g per litre tryptophan, the precursor for indole compounds. Medium was poured into sterile Petri plates and allowed to cool. Once cool, sterile paper filter disks were placed over the media and spores were inoculated onto the top of the filter disk. After 1 wk of growth the filter disks were removed and placed into a Petri plate containing 20 mL of Ehrlich reagent. Disks were allowed to rest for 5 min then colour changes were noted. If no colour changes were noted the test was considered negative: the fungus did not produce significant quantities of indole-type compounds as secondary metabolites. A red or purple colour change was considered positive for indole-type compounds.

2.7. Other tests

2.7.1. Nutrient requirements of TSTh20-1

A nutrient-free growth medium was created by mixing 15 g molecular grade agarose with 1 L ultrapure water in a new, unused, bottle to create a 1.5% solution. The medium was autoclaved then allowed to cool to 50 ^{o}C before being poured into sterile Petri plates and allowed to solidify. TSTh20-1 spores were carefully placed in the centre of the media and incubated at room temperature for 48 h. After this incubation, each plate was evaluated for hyphal growth under a dissecting scope. If growth was observed the plates were discarded and a fresh batch of media created.

If no growth was observed, six sterile 5 mm filter paper disks were placed evenly around the outside of the media. Each disk was given 20 μL of one of the following nutrient solutions: 1 M dextrose, 1 M KCl, 1 M Na_2SO_4, 1 M $Ca(NO_3)_2$, 1 M Na_2HPO_4, standard micronutrients solution (Kaminskyj, 2001). Two additional replicate plates were created randomizing the location of each nutrient around the outside of the plate. Six more sets of plates, with 3 randomized replicates of each, were created excluding one nutrient solution from each. Plates were incubated for 1 wk, after which the plates were evaluated for hyphal growth, sporulation, and direction of growth under a dissecting scope.

2.7.2. pH changes in media induced by TSTh20-1

pH changes induced in the growth media by TSTh20-1 were observed by mixing 5 mL of 25 mM bromocresol purple per litre of medium. Medium pH was adjusted to the neutral point of bromocresol purple, ~ pH 6. Medium was autoclaved then poured into sterile Petri plates. TSTh20-1 spores were inoculated at the edge the side of the plate, allowing maximum distance to observe gradients. The growing colony was imaged once every 24 h for 1 wk. Colour changes and patterns were noted in the media.

2.7.3. Compatibility with tree species

Jack pine seeds were generously donated by Coast-to-Coast Reforestation (http://c2ctrees.com/). Magenta boxes were prepared with TS as previously described. Five jack pine seeds were planted into each of 10 Magenta boxes. Five boxes were inoculated with 10 charcoal pellets containing TSTh20-1 and the remaining 5 boxes were mock inoculated with sterile charcoal pellets. Plants were grown in a phytotron chamber for 3 months under a 16:8 h light dark cycle, 20 ^{o}C:11 ^{o}C day night temperature regime, and constant 80% relative humidity. These conditions are representative of Athabasca region in early summer (Environment Canada). At the end of 3 months, seedlings were carefully removed from the boxes and separated into roots and shoots. Root length, shoot length, wet root biomass, wet shoot biomass, dry shoot biomass, dry root biomass, and needle length were measured for each tree. The student's t-test was performed to determine significant differences in growth.

2.7.4. Demonstration of reduction in soil hydrophobicity by Tween 20 solutions

Soil hydrophobicity was found to be one of the conditions effecting plant growth on TS, creating conditions where water is extremely limiting to the plant. Two simple experiments were performed to demonstrate the effectiveness of using a mild surfactant to reduce this hydrophobicity and enhance plant growth. The first experiment involved filling a glass beaker with hard, cement-like, pieces of TS from Company A. The beaker was filled with water and gently swirled to observe any wetting by water alone. After the water came to rest, a volume of Tween 20 was added to the sand/water mixture to create a ~1% solution (estimated). The beaker was once again gently swirled to mix and the wetting of the sand was observed.

A qualitative test was used to determine if TS treated with TS enhanced or inhibited plant growth. Four pots with ample draining holes were filled with TS, leaving enough room at the top of the pot for watering without overflowing. Two pots were watered with distilled water and the water allowed to drain so that the soil was at field capacity. The remaining two pots were watered with a 1% solution of Tween 20 and allowed to drain to field capacity. Each pot was seeded with 10 radish seeds and allowed to grow for 1 week with no additional watering. After one week the germination and health of each pot was recorded by photo.

3.0 RESULTS

3.1. Soil conditions

To the best of my knowledge, this is the first report on soil conditions found in Alberta oil sand coarse tailings in the public domain. Tailings from two companies were compared. Company A uses sodium citrate in their slurry rather than NaOH, and they do not add a flocculent (see below) to their tailings stream to consolidate tailings. Company B uses the more common method of adding NaOH to their slurry and they add gypsum to their tailings stream as a coagulant (Bordenave *et al*, 2009). These different processing methods may account for the differences in coarse tailings conditions.

3.1.1. Company A

Tailings from Company A were tested for major soil nutrients, organic carbon, pH, hydrophobicity, and total petroleum hydrocarbons (TPH). TS had no detectable nitrogen (total or ammonia), phosphate, or potassium. Low levels of sulfate were present, ~8 ppm. Low levels (~5 %) of organic carbon were present in all samples, as well as low levels of organic matter (< 1 %).

TPH are divided into 4 fractions as per the Canadian Council of Ministers of the Environment (CCME) guidelines (CCME, 2008). These are F1 (benzene, toluene, ethyl-benzene, and xylene [BTEX]), F2 (C10-C16), F3 (C16-C34), and F4 (C34-C50). No detectable amounts of BTEX were found in soil samples. For the others, F2 was < 30 ppm; F3 was 319 ppm; F4 was 197 ppm. The total of these fractions, TPH was 516 ppm. See Figure 3.1.1 for a graphical representation of TPH values. Untreated TS were found to be hydrophobic, as previously reported by Bao (2009). Soil pH was found to be moderately alkaline per US EPA standards, ~pH 8.

3.1.2. Company B

Tailings from company B were found to have no detectable amounts of nitrogen (total), phosphate, or potassium. High levels of sulphate (~250 ppm) were observed in these samples, most likely due to the addition of gypsum to the tailings stream. No detectable amounts of BTEX were found in soil samples. Fractions 2-4 were as follows: F2, < 30 ppm; F3, 319 ppm; F4, 162 ppm for a TPH of 481 ppm. See Figure 3.1.1 for a graphical representation of TPH values. Untreated TS were hydrophobic. Soil pH was extremely acidic, ~pH 3.1.

3.2. Application methods

Effective deployment of a microbial inoculant resulting in a high level of plant colonization is an essential step in development of the inoculant for application in a revegetation strategy. Different microbes might require particular application techniques, so several were tested to find the most effective.

TSTh20-1 was very aggressive in its ability to colonize plants, at least in Magenta box experiments. Regardless of application method tested, plants were always colonized. Furthermore, TSTh20-1 was found growing in both potting mix and in TS regardless of the presence of plants (Figure 3.2.1). Fungal hyphae and spores that morphologically resembled TSTh20-1 could be observed growing in the soil a few days after inoculation. This aggressive growth pattern resulted in plant colonization even if added to the soil >9 cm away. Axenic plants grown alongside experimental ones were not colonized with any endophyte, verifying the effectiveness of soil sterilization techniques and that the endophyte colonizing the plant was the applied endophyte only.

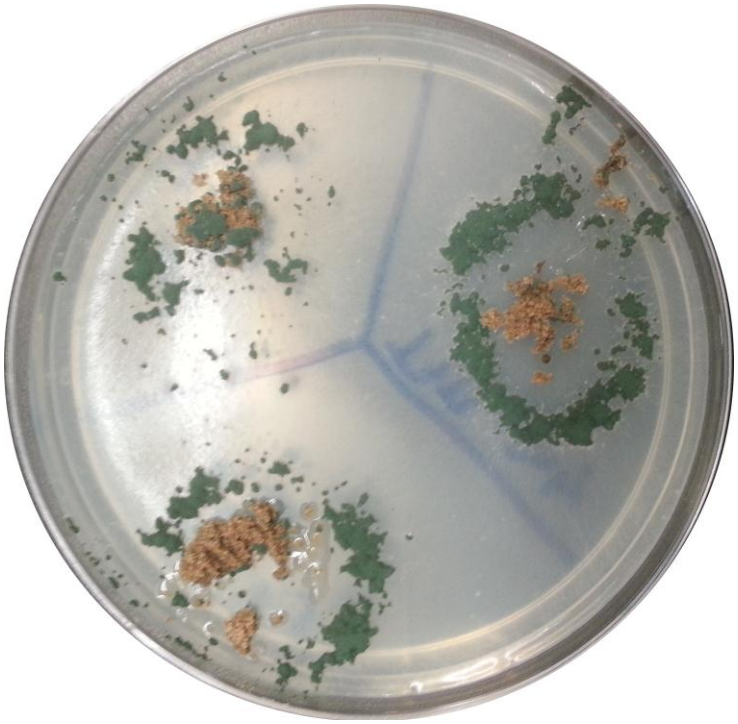

Figure 3.2.1. TSTh20-1 growing from TS samples that were inoculated with the endophyte but had no plants grown on them for 1 month.

3.3. Abiotic stress assays

3.3.1. Drought tolerance and recovery

The hydrophobic nature of TS makes water a limiting resource for plant growth, meaning any increase in the ability to utilize limited water resources could be very benificial. TSTh20-1

enhanced drought recovery in tomato plants that had been allowed to wilt for 36 h and then drenched. After just 15 min of recovery, the plants colonized with TSTh20-1 had significantly higher leaf water content ($P = 0.0099$). This relative water content continued beyond 30 min ($P = 0.0077$). Axenic plants showed a linear of recovery ($r^2 = 0.981$) whereas plants colonized with TSTh20-1 showed a logarithmic recovery curve ($r^2 = 0.9988$). The difference in plant water content and apparent turgor was visible by 30 min. See Figure 3.3.1 for a graph of leaf water contents with their best fit lines, as well as an image of plants 30 minutes into their recovery. No significant differences were recorded during the desiccation period, suggesting TSTh20-1 does not assist with drought tolerance. However, the slightly higher t=0 water content in TSTH-colonized vs axenic plants suggests a trend toward improved water use efficiency.

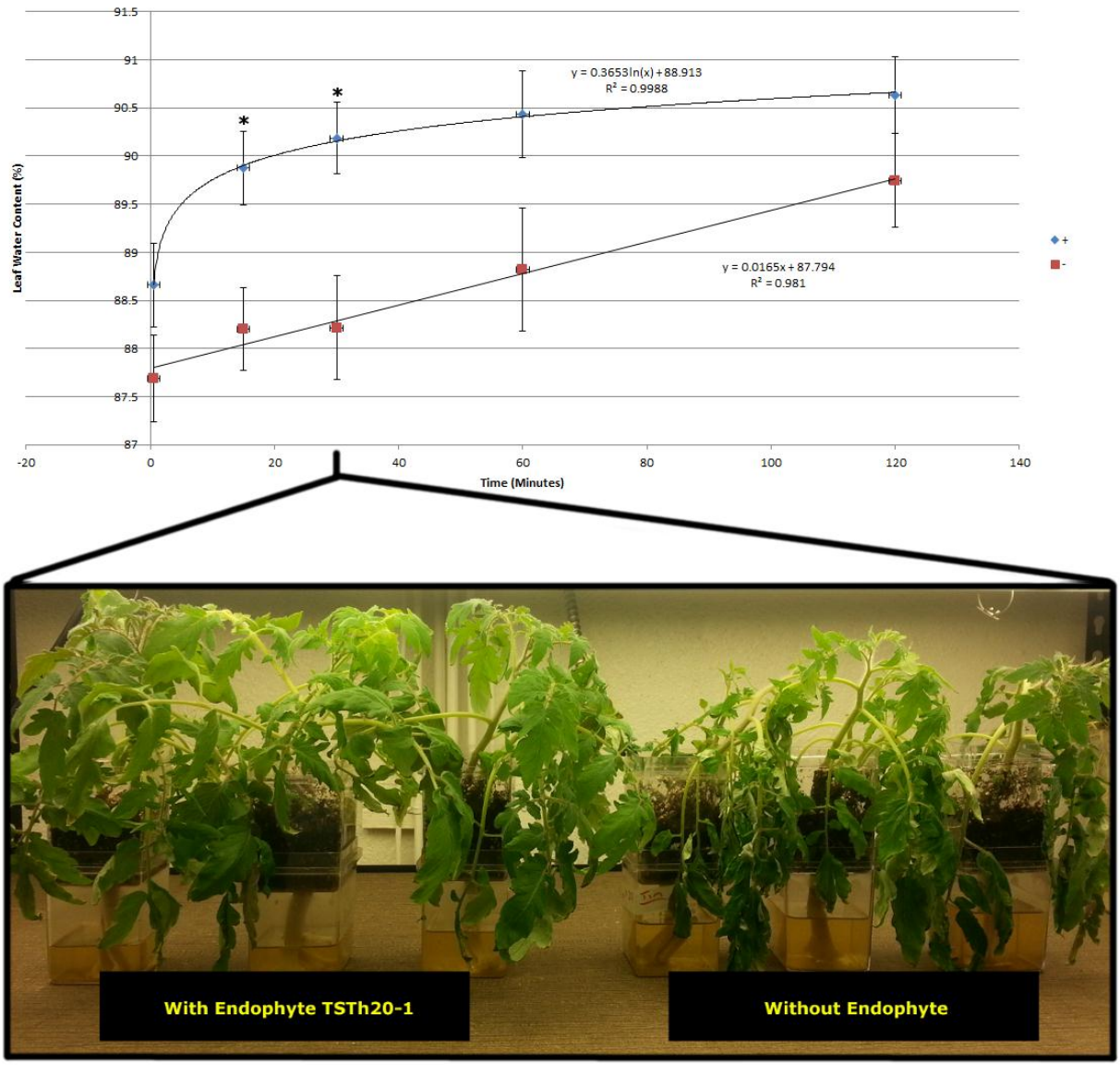

Figure 3.3.1. The drought recovery curves of axenic and inoculated plants. Leaf water content was measured 5 times over the course of drought recovery and plotted. Blue diamonds (upper line) represent plants grown with TSTh20-1 while red circles (lower line) represent axenic plants. Significant time points are denoted with an asterisk. The lower half of the figure shows visually the difference in drought recovery 30 minutes into recovery.

3.3.2. Seed germination assays

Environments with stressful conditions will result in lower germination rates and higher rates of seedling mortality. Some strains of *T. harzianum* have been shown to enhance seed germination rates. TSTh20-1 was found to enhance germination rates of clover seeds grown both on potting mix ($P < 0.01$) and TS. A P-value was not recorded for growth on TS, however figure 3.3.2 shows the visible differences in growth on potting mix. Figure 3.3.2 shows the visible differences in germination rates of clover seeds grown on PM for 7 d.

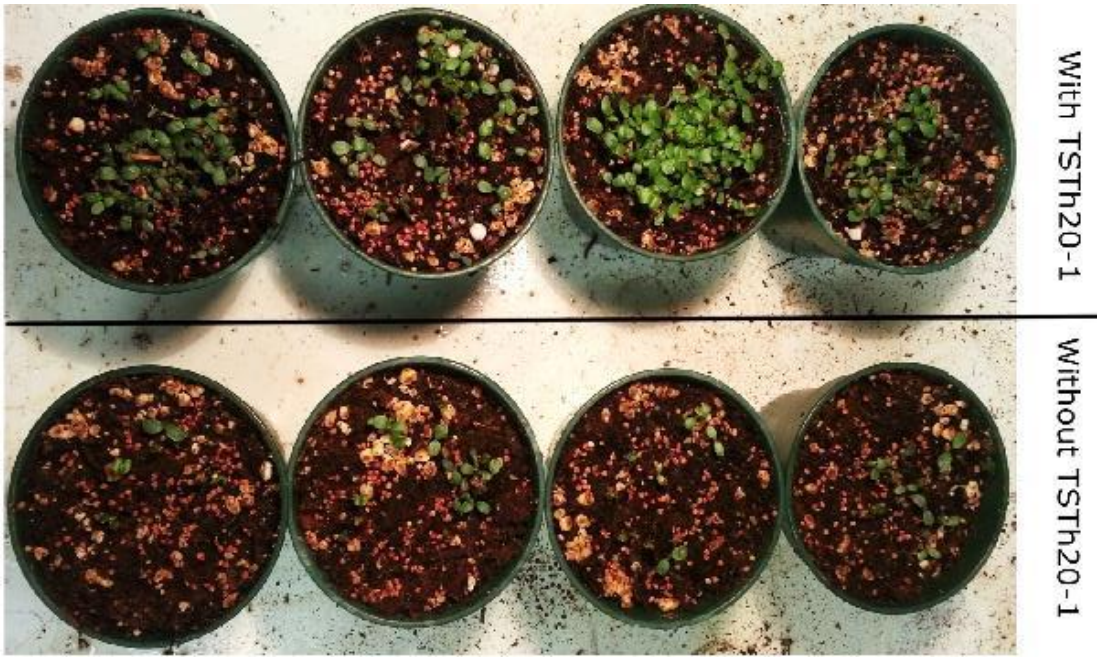

Figure 3.3.2. Seed germination enhancement of white clover (*T. repens*) by TSTh20-1 on potting mix after a 1 w growth period.

3.4. Selection of native species

3.4.1. Growth on tailing sands

Choosing the appropriate species for reclamation can be difficult. Avoiding invasive species, plants that can survive the difficulties of the environment, and plants that require limited maintenance, such as fertilizer and watering are needed. The 24 species screened for growth on TS were grouped into 3 categories based on qualitative inspection. These were strong growth on

TS; weak growth on TS; no growth on TS. Figure 3.4.1 shows examples of strong and weak growth on TS. Generally, seeds requiring cold stratification either did not germinate, or the seedlings grew poorly. This may be partially due to a longer exposure to toxins found in TS, or their smaller seed size (*data not shown*) making them more vulnerable to small amounts of toxic materials.

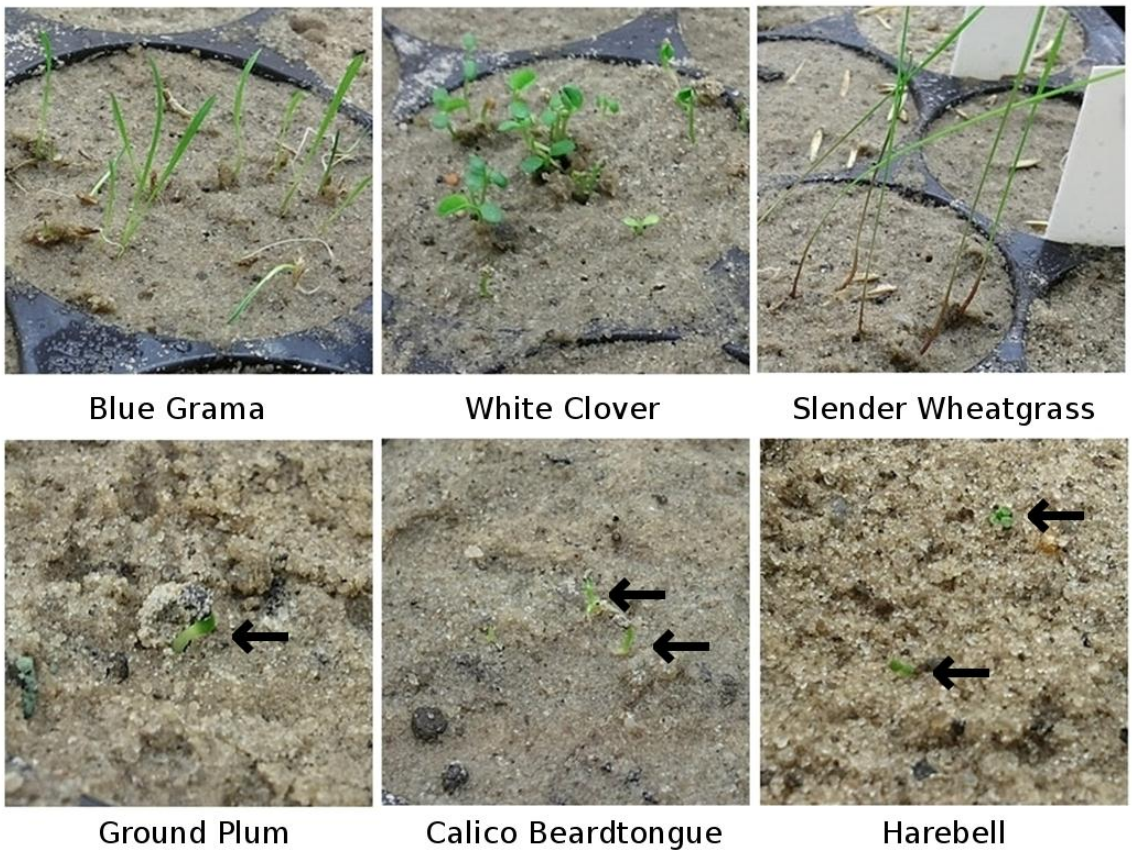

Figure 3.4.1. Some examples of strong and poor growth of native species on TS. Top row (Blue grama, White clover, Slender wheatgrass) show strong growth on TS. Bottom row (Ground plum, Calico beardtongue, Harebell) show weak growth on TS. Arrows indicate location of small seedlings.

3.4.2. Seed mixture

Monoculture of a species, even in reclamation, can present problems in the event of a dynamic environment, disease, or insects that could result in mass mortality of monocultures. A

diversity of species can result in lower maintenance of a site by decreasing the risk of catastrophic mortality from a single cause. Four plants species were used in a seed mixture for microcosm revegetation studies. They were chosen for their strong growth on TS and suitability to reclamation purposes: stabilizing soil, adding nutrients to soil, and rapid establishment. The four species chosen were: blue grama (*Bouteloua gracilis*), slender wheatgrass (*Elymus trachycaulus*), alfalfa (*Medicago sativa*), and white clover (*Trifolium repens*).

3.4.3. Competition effects and compatibility with TSTh20-1

When using a mixture of seeds it is important to ensure that competition effects between species are not hindering overall growth. No competition effects were noted regardless of seeding density on limited volumes of TS after the growth period. Qualitative analysis suggests that plants grown in higher densities were performing as well as, or better than, plants grown in lower seed densities as shown in Figure 3.4.3. This may be due to the extremely low nutritional availability in TS, leaving little for plants to compete for. It may also be that plants grown in higher densities better distributed the toxic load of residual hydrocarbons and moderate pH levels amongst more plants leading to better performance as a whole despite limited nutrients, however there is no data to support either of these hypotheses. Over a longer growth period competition effects may become more apparent as pH and toxins are reduced.

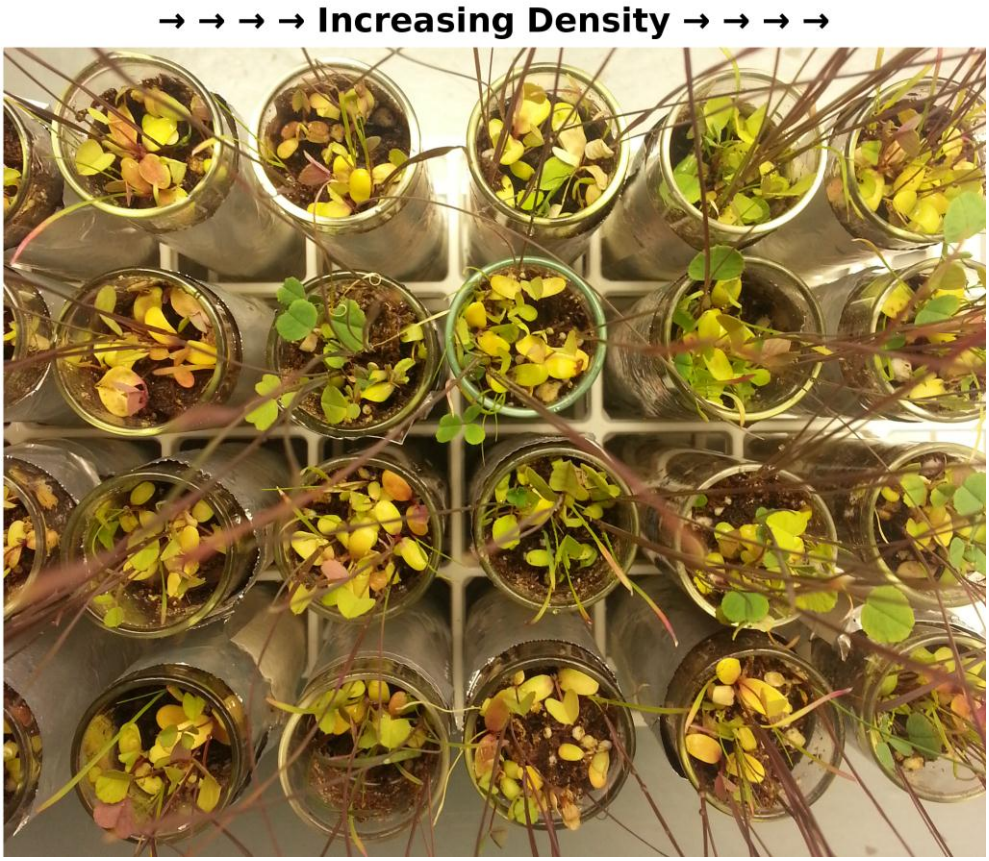

Figure 3.4.3. Seed density experiment as shown from above. Seeding density increases from left to right. No dramatic differences in plant growth were apparent after the 4 wk growth period.

3.5. Mesocosm scale experiments

3.5.1. Tailing sands from Company A

To investigate the potential effects of plant growth and soil conditions on a larger scale, a mesocosm scale experiment was established. Twelve PVC tubes (45 cm tall x 10 cm diameter) were set up. Half of the tubes were filled with TS and the other half PM. All tubes were seeded with a seed mixture, and 3 tubes from each group inoculated with TSTh20-1. Plants were allowed to grow, with regular watering, for 2 months. Plant parameters such as shoot length, root length, fresh and dry biomass, and root to shoot ratios were measured. No significant differences in plant health parameters (root length, shoot length, wet biomass, dry biomass, and root:shoot ratios) were observed after 2 months of plant growth ($P > 0.05$). Plants were verified for axenic

or colonized conditions. Axenic plants were found to be sterile with the exception of an orange endophyte found in blue grama seeds that eventually colonized other plants, both monocot and dicot, as well. This orange endophyte was observed growing from > 90 % of seeds on Petri plates. It is believed that it is a Class 1 fungal endophyte that could not be removed via surface sterilization. It was found that TSTh20-1 and the putative Class 1 endophyte both colonized plants grown on TS (Figure 3.5.1.1) Nutrient levels in the soil were found to be unchanged as all nutrients remained below detectable levels.

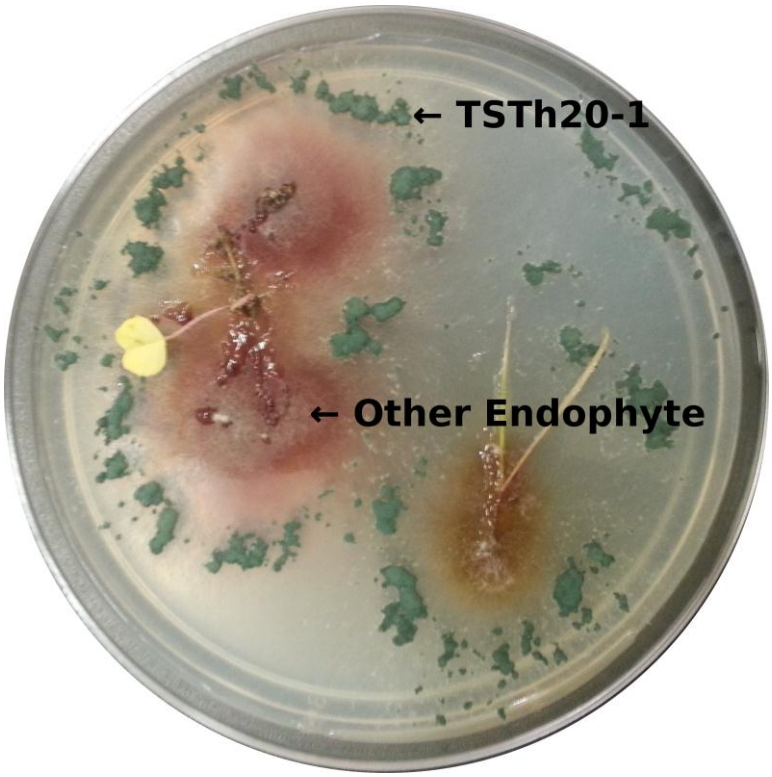

Figure 3.5.1.1. A Petri plate containing surface sterilized plants that were grown on TS in the presence of TSTh20-1 for 1 month. Both TSTh20-1 and the orange endophyte from the blue grama seeds can been seen growing from a dicot (Alfalfa) and monocot (Blue Grama).

TPH levels in TS *increased* from 510 ppm to > 800 ppm with the growth of axenic plants on the TS for two months. Hydrocarbon levels *increased* to nearly 1400 ppm with the growth of plants inoculated with TSTh20-1 on TS for two months. Figure 3.5.1.2 shows the increase in

hydrocarbons over the baseline level. The significance of this increase cannot be determined as only one sample was analyzed which was a mixture of three replicate mesocosms. Hydrocarbons were not expected to increase, see the discussion for a hypothesis as to why this might have occurred.

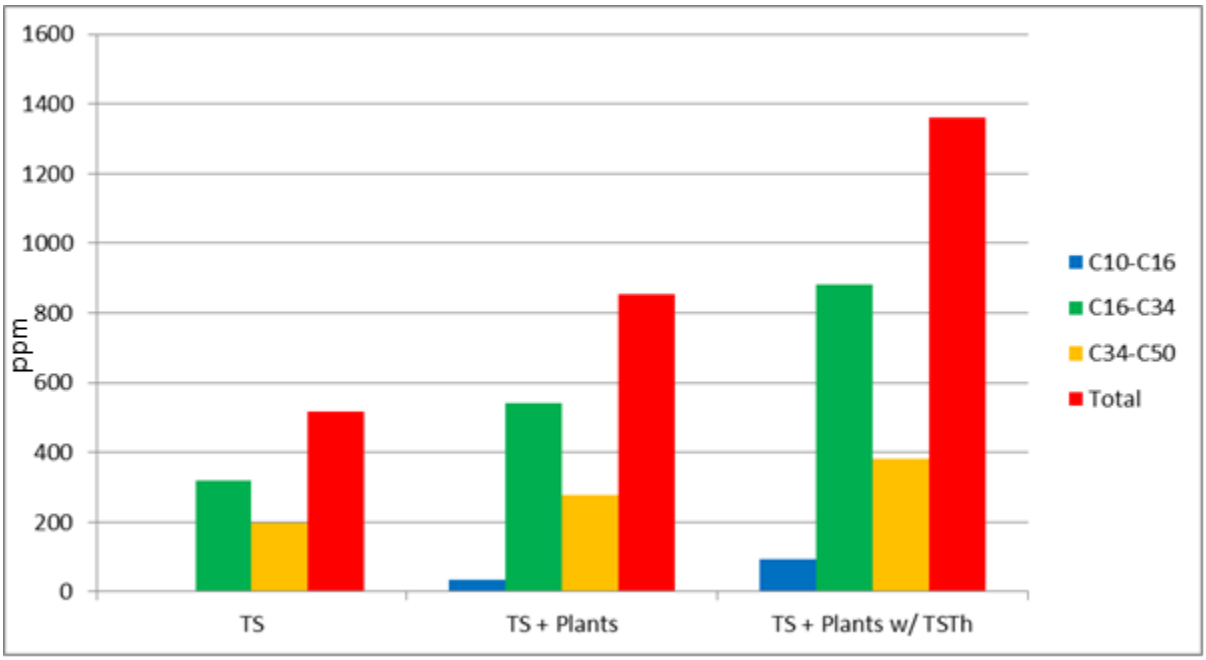

Figure 3.5.1.2. A graph showing the increase in hydrocarbons observed in the Company A mesocosm experiment. Hydrocarbons are broken shown as 3 CCME fractions (F2, blue; F3, green; and F4, yellow) as well as total hydrocarbons (red). *Left* shows the baseline level of hydrocarbons observed in TS, *centre* shows hydrocarbon levels when axenic plants were grown on TS for 2 months, and *right* shows hydrocarbon levels after plants inoculated with TSTh20-1 were grown on TS for 2 months. Hydrocarbon levels increase in the presence of plants, and greatly increase when TSTh20-1 was added.

3.5.2. Tailing sands from Company B

A second mesocosm-scale experiment using tailings from Company B, due to limited quantities of tailings from Company A, was designed to test why dramatic increases in measureable hydrocarbons were observed in the first experiment. Soil hydrocarbons were

measured in randomly selected replicates. No significant differences were observed in TPH levels between treatment groups in the F2, F3, F4, or TPH ($P > 0.100$). Soil peroxidase activities were found to not increase with the addition of TSTh20-1 to TS without the presence of plants. However the presence of plants alone significantly increased peroxidase activities ($P < 0.05$). Plants inoculated with TSTh20-1 increased peroxidase activity significantly over the control and plants-only treatments ($P < 0.05$). Please see the discussion section on possible reasons why differences in hydrocarbons were not observed in this experiment. The orange endophyte observed in blue grama and the Company A experiment was observed in this experiment as well.

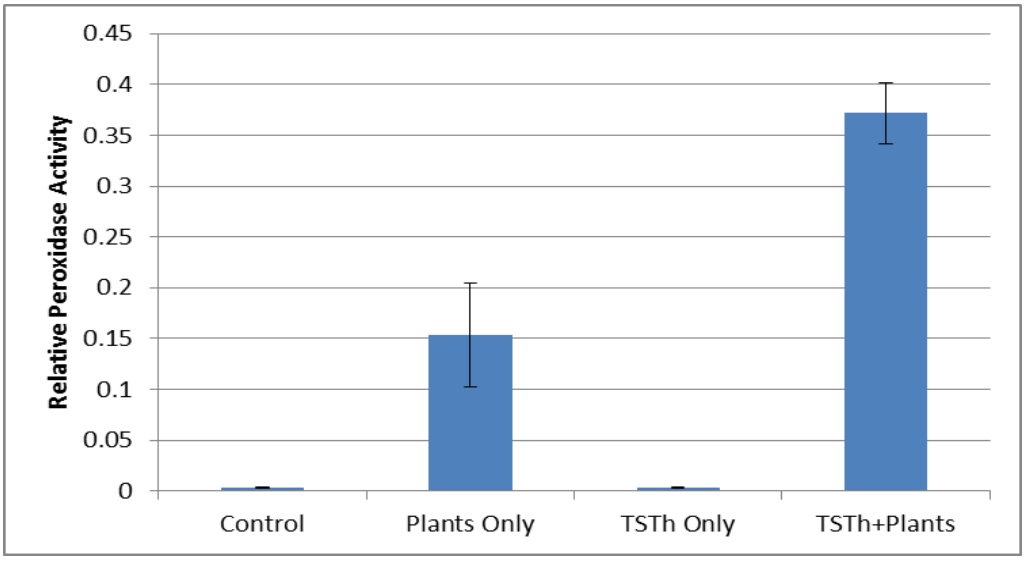

Figure 3.5.2. Relative levels of cell-free peroxidase activity in TS under a variety of treatments. Soil peroxidase activity was found to increase under the presence of plants, and increase significantly more when TSTh20-1 inoculated plants were present. The addition of TSTh20-1 alone to TS did not increase peroxidase activity.

3.6. Mechanisms of plant growth promotion

3.6.1. Phosphate solubilization and organic acid production

The nature of TS dictates a need for any organism(s) living on it to be an excellent scavenger of the few nutrients present. Phosphate, which is essential and known for its limited solubility in soil, was chosen for this study. TSTh20-1 strongly solubilized Al-phosphate precipitate in liquid culture. Solubilization of this solid was as strong, or stronger than, the

solubilization observed with *Penicillium bilaiae*, a known phosphate solubbilizer. Solubilization of Ca- and Fe-phosphate precipitates was weak relative to *P. bilaiae*. Using HPLC, TSTh20-1 was found to produce oxalic, gluconic, and citric acids, which together are likely responsible for the solubilization of phosphate solids observed in liquid culture via pH changes and anion exchange. Figure 3.6.1 shows the relative levels of PSOL performed by both TSTh20-1 and *P. bilaiae* on three types of phosphate precipitates in liquid culture.

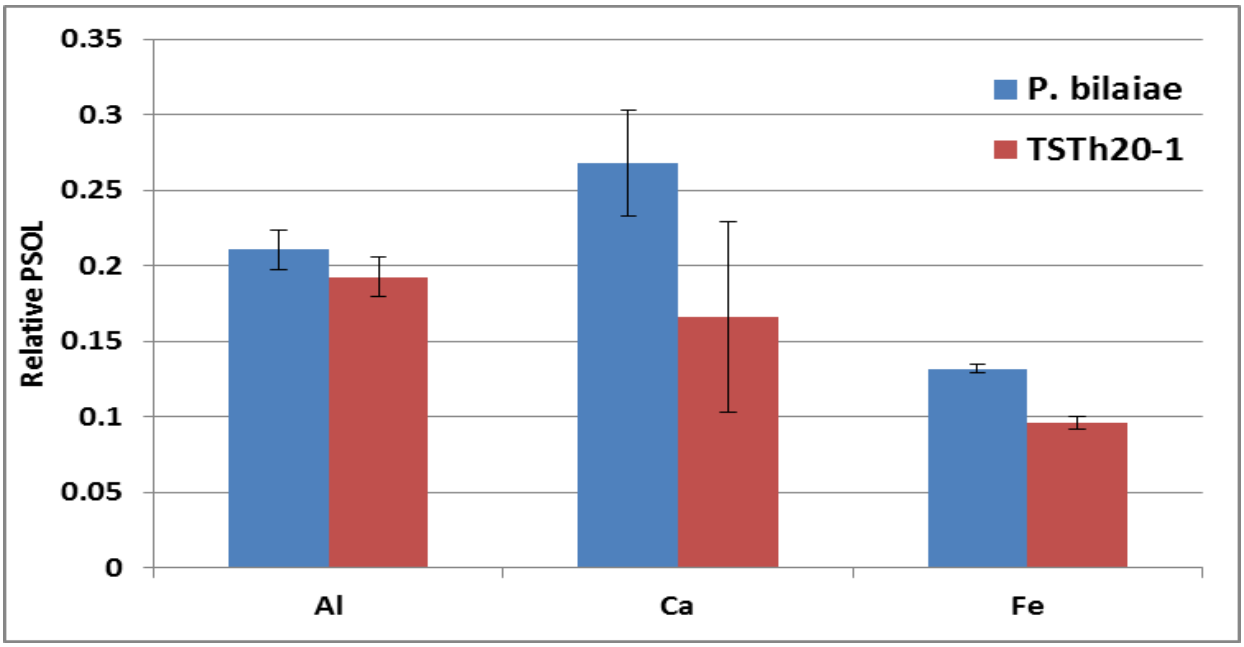

Figure 3.6.1. Relative levels of PSOL, represented as optical density, of *P. bilaiae* and TSH20-1. PSOL capability of each fungus was measured in liquid culture with insoluble phosphate precipitates (Al, Ca, and Fe) for 1 wk.

3.6.2. Production of siderophores

Siderophores have been implicated as one possible mechanism used by PGP bacteria to enhance plant growth, it stands to reason the PGP fungi may also utilize these compounds. However, TSTh20-1 did not produce detectable levels of siderophores *in vitro*. This was compared to *P. bilaiae*, which produces large amounts of siderophores that can be visually detected on CAS media. Since this test was not performed using a plant, it is impossible to say whether TSTh20-1 produces siderophores or induces siderophore production *in planta*.

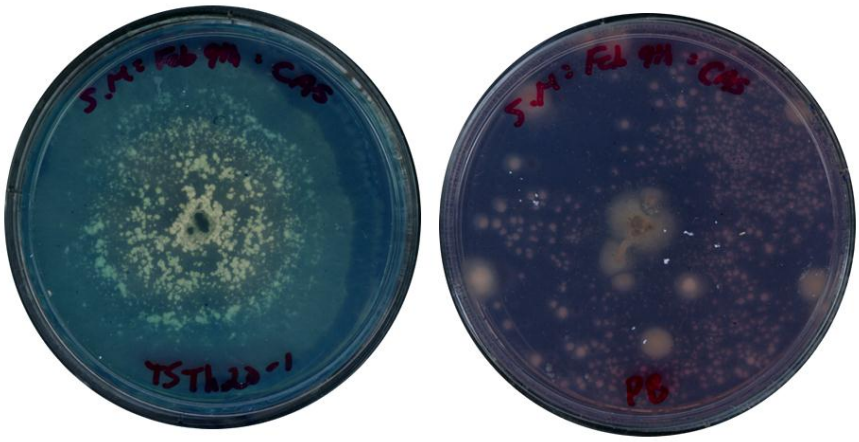

Figure 3.6.2. CAS plates showing siderophore assay for TSTh20-1 and *P. bilaiae*. TSTh20-1 (left) shows no colour change in the media, an indication that no siderophores are produced. *P. bilaiae* (right) shows a change in the media from blue to pink, a positive reaction for siderophore production.

3.6.3. Tolerance to reactive oxygen species

Under stress conditions, many plants produce reactive oxygen species (ROS), that can lead to cell damage and death. Some fungal endophytes have been shown to protect plants from this effect by unknown mechanisms. TSTh20-1 did not protect plants from the generation of ROS under stressed (grown on TS) or non-stressed (grown on PM) conditions. Leaf disks taken from plants grown under different conditions were found to bleach at similar rates regardless or endophyte colonization.

3.6.4. Hormone production

Some fungal endophytes and PGP bacteria produce plant hormones that can lead to growth changes in the plant. Common plant hormones include IAA, and other indole family compounds. TSTh20-1 did not produce indole family compounds *in vitro*. TSTh20-1 may produce other types of hormones that were not tested for such as jasmonic acid, ethylene, or gibberelins. Since this test was not performed using a plant, it is impossible to say whether TSTh20-1 produces hormones or induces hormone production *in planta*.

3.7. Other tests

3.7.1. Nutrient requirements of TSTh20-1

Given the oligotrophic nature of TS, it was hypothesized that TSTh20-1 would have low nutrient requirements (Kaminskyj *et al*, 2008). Low nutrient requirements would also place minimal stress on the host plant. TSTh20-1 was found to grow in extremely oligotrophic environments. TSTh20-1 would readily grow on media prepared with nothing but molecular grade agarose and 18 MegOhm ultrapure water. Growth was weak, but viable spores were produced even under these conditions. This growth and spore production may be part of the dowry effect, where nutrients stored in spores are used for growth and further sporulation in absence of nutrient in the environment. Only one batch of molecular grade agarose did not support growth of TSTh20-1. Spores did not germinate on media made from this agarose, so it was used to test for the nutritional requirements for spore germination and hyphal growth. TSTh20-1 spores did not germinate without the presence of micronutrients found in standard micronutrient solution. Germling growth showed a strong tendency to grow in the direction of micronutrients, regardless of micronutrient position on the plate. Tests were not performed to determine which micronutrient was the most limiting for fungal spore germination and growth. This ability to grow in oligotrophic conditions is reflective of the oligotrophic environment found in TS.

3.7.2. pH changes in media

The effect of TSTh20-1 on medium pH was examined as a control for organic acid production (e.g. if acids are not produced the media will remain neutral). TSTh20-1 demonstrated unexpected changes in medium pH when grown on PDA. pH was found to increase near the youngest hyphal tips for 20-50 mm, older hyphae strongly acidified the media, and hyphae associated with sporualtion made the medium strongly alkaline. Figure 3.7.2 shows the pH changes induced in media as TSTh20-1 grows.

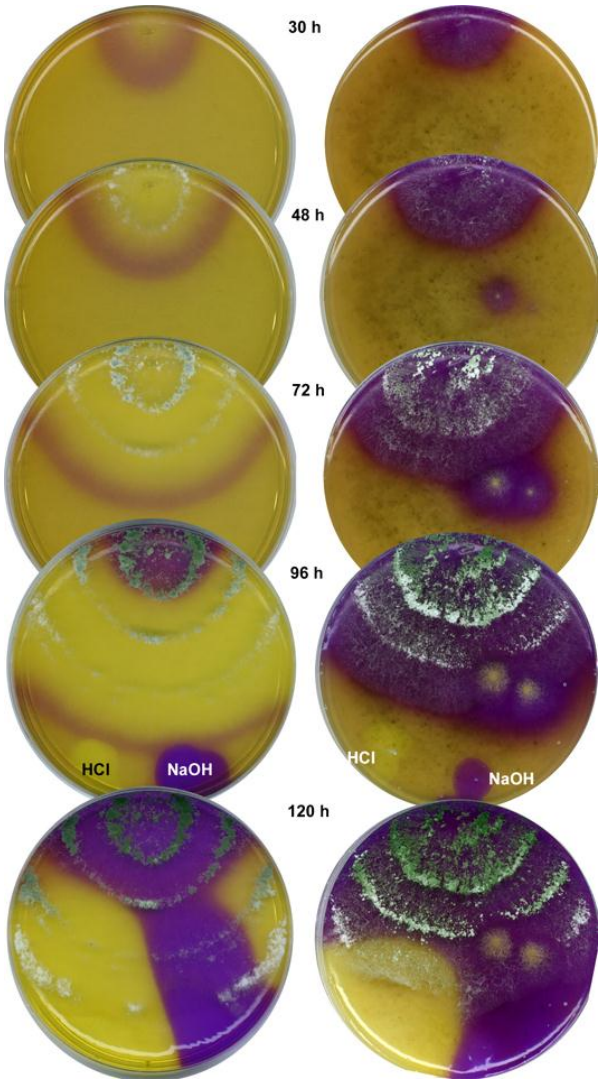

Figure 3.7.2. pH changes induced in 2 media by TSTh20-1 as indicated by bromocresol purple. *Left*: TSTh20-1 grown on 10 % PDA for 120 h. A bull's-eye effect of high-low-high pH can be observed in the media. *Right*: TSTh20-1 grown for 120 h on medium containing an insoluble phosphate source, bone meal. The pH patterns are not similar to those seen on 10 % PDA. HCl and NaOH are used as controls.

3.7.3. Compatibility with trees

The legal requirements of reclamation in Alberta dictate that the affected land must be restored to an equivalent land capacity before a reclamation certificate can be awarded; in this case that is a mixed wood boreal forest. As such, knowing the effects of TSTh20-1 on tree growth could be important for future work. TSTh20-1 was found to inhibit the growth of Jack pine seedlings when grown on TS, particularly the shoot section of the plant. Trees inoculated with TSTh20-1 had shorter needles ($P = 0.002$), shoots ($P = 0.023$), and lower shoot biomass ($P = 0.006$). No significant differences were observed in length of root ($P = 0.129$), root branching ($P = 0.124$), or root biomass ($P = 0.424$). The reason for these differences was not studied, however may be attributed to increased levels of short-chain hydrocarbons (See Discussion). Figure 3.7.3 shows representative plants from each treatment group.

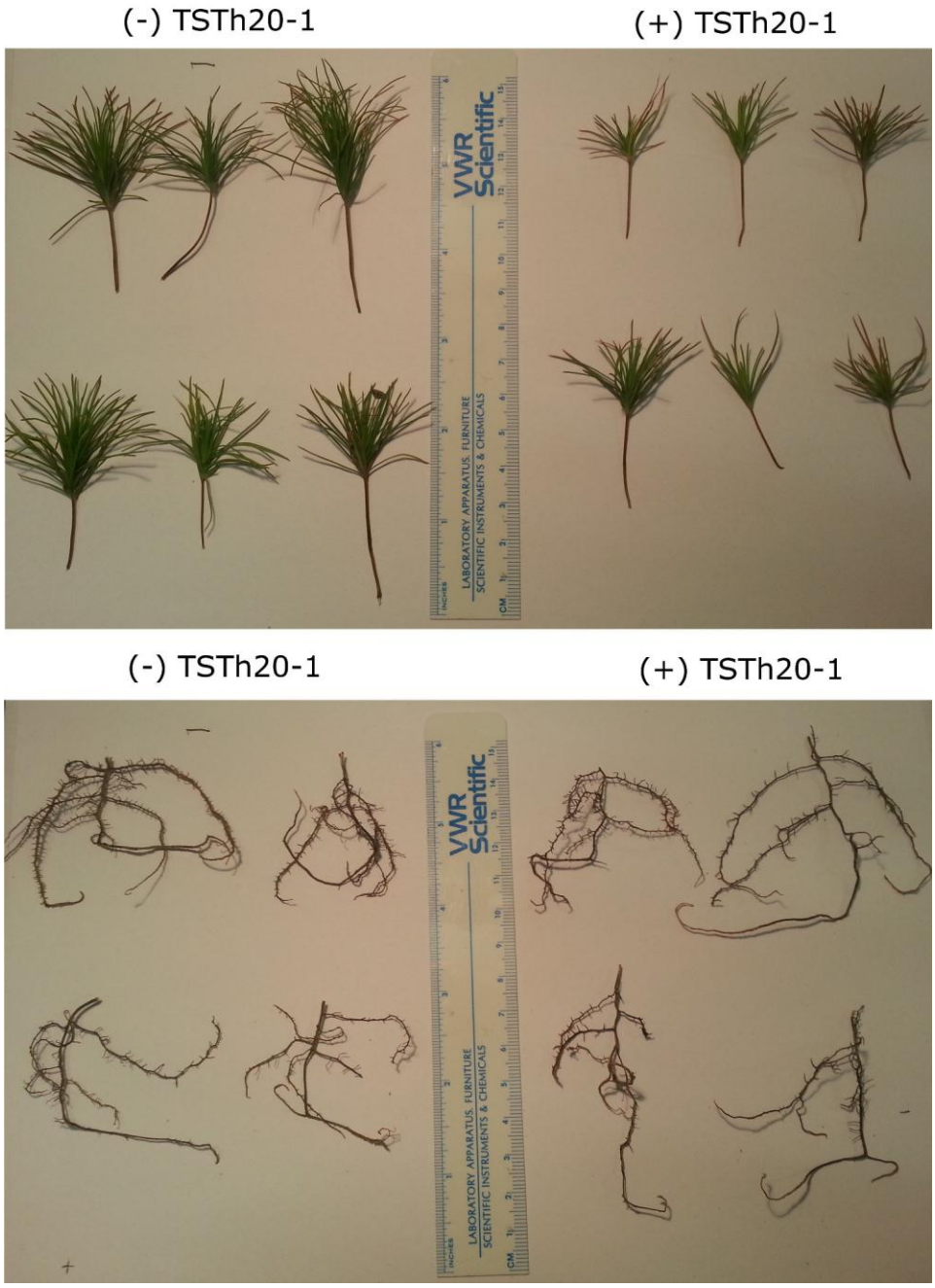

Figure 3.7.3. Growth differences observed in 2 month old jack pine seedlings grown on TS with and without TSTh20-1. No differences were observed in root growth parameters. Shoot growth was inhibited in trees grown with TSTh20-1.

3.7.4. The effect of surfactants on soil hydrophobicity and plant growth.

Previously (Bao, 2009), TS have been shown to be strongly hydrophobic. Soil hydrophobicity became a pronounced problem when establishing experiments throughout this study. TS from Company A in their raw, dry, form were found to be cement-like in nature and strongly hydrophobic. Aggregates of this hard and dry TS could be left in water for extended times without the sand wetting. Similarly, in experimental setups, pots containing TS were watered; the water would remain on the surface of the sand, never soaking in to the sand aggregates. Treating the TS with a 1% solution of Tween 20 was found to eliminate soil hydrophobicity without the need for repeated treatments (Figure 3.7.4.1). In a second experiment to determine the effect this has on plant growth it was found that radish seedlings growing on Tween 20 treated TS were still alive after one week of no watering, while those grown on raw TS were wilted beyond saving. The soil in each pot also reflected this; the treated soil was homogenous in nature and still moist after one week while the untreated TS were completely dry and still contained numerous hard sections (Figure 3.7.4.2).

Figure 3.7.4.1. A demonstration of the effect Tween 20 has on TS hydrophobicity. The image on the left shows tailing sands in water after gentle mixing. The tailing sands aggregates remain dry. The image on the right shows the same tailing sands after the addition of Tween 20. The tailing sands are easily wetted after this.

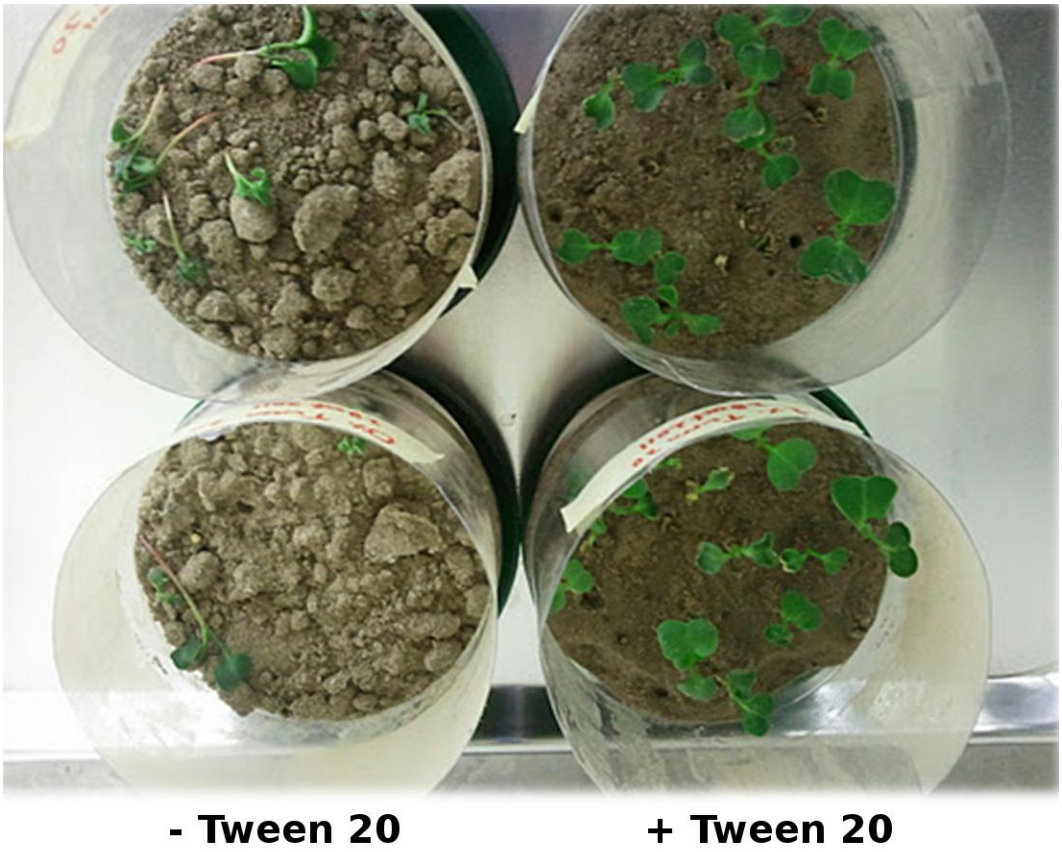

Figure 3.7.4.2. A preliminary experiment testing the effects of Tween 20 treated TS on plant growth. Left: Seedlings grown on untreated TS perish due to water stress. TS are visibly dry and clumped. Right: Plants on treated TS are not water stressed. TS are visibly wet and not clumped.

4.0. DISCUSSION

4.1. Tailing sands are an environment of extremes

Tailing sands are a difficult environment for plants to survive on, let alone thrive. Regardless of extraction method, TS were found to contain no detectable amounts of nitrogen, phosphate, or potassium. These are the three most essential inorganic macro-nutrients required for healthy plant growth. Without available NPK, plants must dedicate more resources to obtain the necessary amounts, for example producing longer or larger root systems.

TS have been shown to be hydrophobic in both this and previous studies (Bao, 2009). TS hydrophobicity means that water will not permeate into the pore spaces of aggregates, thusly limiting the amount of water plants can use for their growth. TS hydrophobicity creates problems for current accepted methods of reclamation. These include increased water runoff, possibly leading to erosion of the peat mineral mix used to cap tailings. TS hydrophobicity may play a role in inhibiting the growth of trees and larger plants on the peat mineral cap because their root systems could extend into the hydrophobic TS where water relations will be affected, potentially limiting root growth. TS from Company A contained barely detectable levels of sulfate, whereas TS from Company B contained large amounts. This is most likely due to the addition of gypsum to the tailings stream by Company B. Gypsum serves as a coagulant that causes fine tailings to settle at an increased rate (Devenny, 2009). With the exception of sulfate, no detectable amounts of NPK, nutrients key for plant growth, were found in TS from either company. Without the addition of fertilizer or an endophyte that assists in nutrient acquisition, possibly both, uptake plant growth with be severely limited.

Table 4.1. A summary of conditions found in coarse tailings from two companies.

Test	Company A	Company B
Nitrate	< 4 ppm	< 4 ppm
Phosphate	< 2 ppm	< 2 ppm
Potassium	< 4 ppm	< 4 ppm
Sulfate	8 ppm	250 ppm
Organic Carbon	< 0.8%	Not tested
Hydrocarbons (Total)	516 ppm	481 ppm
Hydrophobicity	Yes	Yes
pH	8	3.1

TS hydrophobicity is thought to arise from residual hydrocarbons coating to the surface of sand particles. One theory is that hydrocarbons are saponified during the extraction process, giving them surfactant like properties (Zhou *et al*, 1999). It is then believed that the hydrophilic

part of the saponified hydrocarbons attaches to water coated sand particles, leaving the hydrophobic side of the hydrocarbons facing outward (Zhou *et al*, 1999). TS hydrophobicity was greatly reduced through a one-time treatment of TS with a 1 % (v/v) solution of Tween 20. Tween 20 is a biodegradable, non-accumulating, surfactant. Surfactants have been used previously to reduce the hydrophobicity of impacted soils to enhance plant growth (Sunderman, 1983). No sources indicate the use of surfactants in oil sands reclamation, this study indicates that they may be useful in reducing or eliminating the hydrophobicity of TS. However, studies should be performed to ensure that surfactants do not increase the mobility and bioavailability of hydrocarbons or other toxins. A proof of concept experiment performed shows the great effect a one-time treatment of Tween 20 can have on water retention and plant growth, leading to enhanced plant growth on TS. This effect is likely due to the increased water availability to the plant.

TS pH was vastly different (~4 pH units) between the two companies. This was directly due to use of different additives to their slurries. TS from Company A were moderately alkaline, whereas TS from Company B were moderately acidic. Both of these pHs fall well outside of the range, pH 4.8 – 6.0, for boreal forest soils in the Athabasca region (Alberta ESRD, 2000). These differences in pH are likely due to the particular additives used by each company in the extraction process.

Company A uses sodium citrate, a weak organic base in their slurry. Due to the low microbial activity in TS (*data not shown*) an organic base is likely to be highly recalcitrant in the soil, which therefore buffers the soil pH at a higher value. Company B uses NaOH, a strong and reactive base, in their slurry. NaOH reacts with CO_2 in the atmosphere to form sodium carbonate, which can form carbonic acid when combined with acidic rainwater. The nominal pH of dissolved carbonic acid at atmospheric levels of CO_2 is ~5.7, not low enough to explain the pH observed. However, the low buffering capacity of carbonic acid may allow other factors present in the soil to lower the pH further to the levels observed. Deviations from normal forest soil pHs may inhibit the healthy growth of boreal forest species. pHs that deviate far from neutral ranges can inhibit the growth of plants that are not adapted (Islam *et al*, 1980).

As expected, TS contained residual hydrocarbons, since the oil extraction process is not 100 % efficient. The level of hydrocarbons found in each fraction was within CCME guidelines for an industrial soil for both companies' TS (CCME, 2008). Figure 4.1 shows an overlay of the TPH profiles from each company. As can be seen the overlays show highly similar profiles of hydrocarbons, with Company B having slightly less Fraction 4 hydrocarbons and more Fraction 2 hydrocarbons than Company A. Both companies have a similar level of total hydrocarbons, around 510 ppm. It is somewhat surprising that the TPH for each company are so similar as each company claims a different efficiency for their extraction methods. Company A claims an extraction rate of 80 % whereas Company B claims an extraction rate of 92 %. Company A is known to reject asphaltenes, large polycyclic hydrocarbons from their extraction process. These are sent to tailings, resulting in a lower extraction percentage. Company B accepts asphaltenes in their extraction process. Knowing this, one would expect to observe a higher percentage of large, Fraction 4, hydrocarbons in the TPH profile from Company A. Asphaltenes are large molecules, many of which contain more than 50 carbons. This is critical since the CCME method for evaluating hydrocarbons does not evaluate the presence of hydrocarbons larger than 50 carbons in size. Thus I expect that the tailings from Company A likely contain a significantly larger amount of hydrocarbons than those from Company B, but due to their size and complexity, they are not detected by the CCME TPH analysis, as this analysis only examines hydrocarbons less than 50 carbons in size. The effect of these large hydrocarbons on plant growth and soil health is not known.

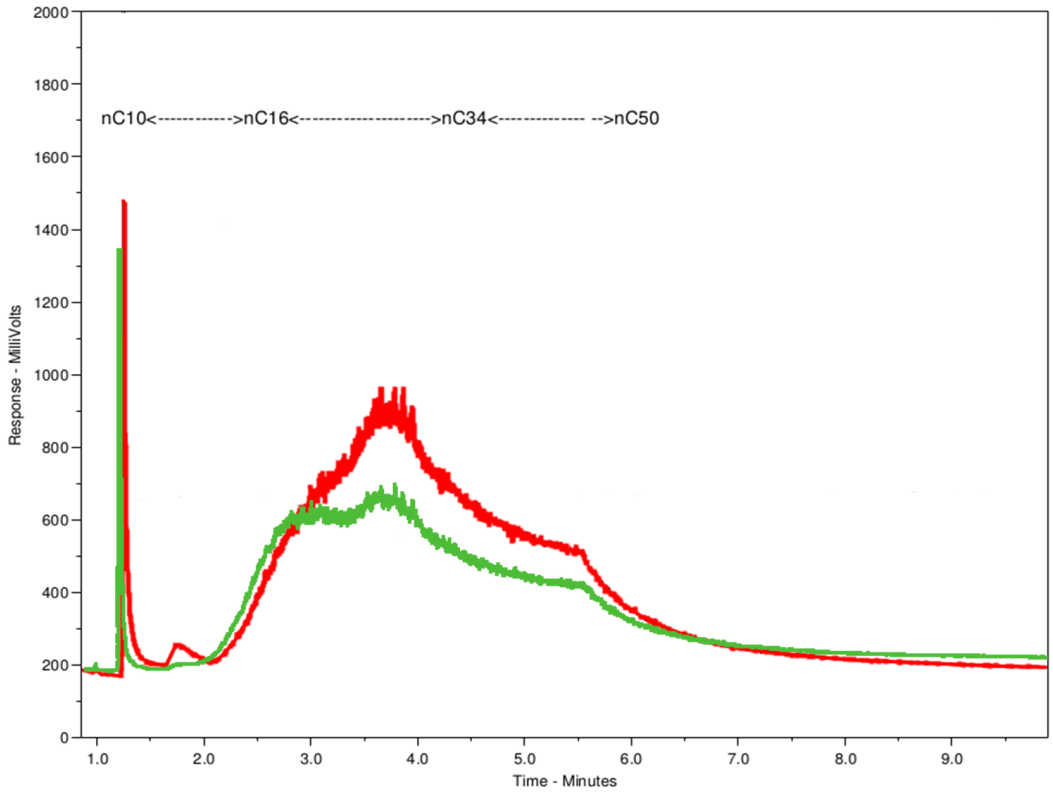

Figure 4.1. Raw chromatograms comparing the relative hydrocarbon levels found in TS from Company A (red) and Company B (green). Company A shows a larger portion of larger hydrocarbons, in line with the extraction methods used by Company A. Company B has a higher ratio of smaller hydrocarbons to larger hydrocarbons.

To the best of my knowledge this is the first report on the conditions found in coarse TS available publically. These results indicate that sustaining plant growth on such an environment is likely to be difficult, and the lack of reclamation certificates issued by the Government of Alberta to-date is in agreement with this assessment.

The most difficult aspects of growth on TS for plants will likely be the limiting levels of plant nutrients and water. No in-depth studies publically available have been performed on coarse tailings to evaluate the presence of toxic compounds or what their effect on plant growth may be. Given the lack of nutrients present in TS, it is possible that inorganic toxins, such as metals, are present in biologically significant quantities relative to organic toxins.

Naphthenic acids (NAs) are a complex group of thousands of related organic compounds, that have been shown to reduce water conductance in woody tree species and are known to be present in OSPW as well as reduce gas exchange and root water transport in aspen (Apostol *et al*, 2004; Kamaluddin *et al*, 2002). However, the concentration of NAs in TS is currently unknown as these have never been measured for solid samples. As it stands, there are no established methods for extracting NAs from soil material. It may be possible that NAs can be extracted from TS using methods commonly used to extract NAs from OSPW, which should be investigated in a future study.

4.2. TSTh20-1, a single endophyte, confers multiple tolerances

Previously, TSTh20-1 was shown to promote the growth of tomato plants on TS (Bao, 2009). The current study tested the ability of TSTh20-1 to promote the growth of native species in medium-scale environments, to enhance seed germination and seedling growth, and to assist in drought tolerance and recovery. TSTh20-1 did not contribute to drought tolerance, but did significantly enhance drought recovery. The ability of TSTh20-1 to assist in water uptake, when water is available, may reflect the hydrophobic environment of the plants from which it was isolated. TSTh20-1 also increased the rate of germination of white clover seeds on potting mix and TS. The mechanism of this germination enhancement is not known. The ability of TSTh20-1 to confer multiple tolerances makes it attractive as a microbial inoculant to be used in environments where those stresses are found. However, the scope of the tests performed is limited to few soil types and in highly controlled conditions. Further testing under a larger range of circumstances should be done before TSTh20-1 can be recommended for wide spread use.

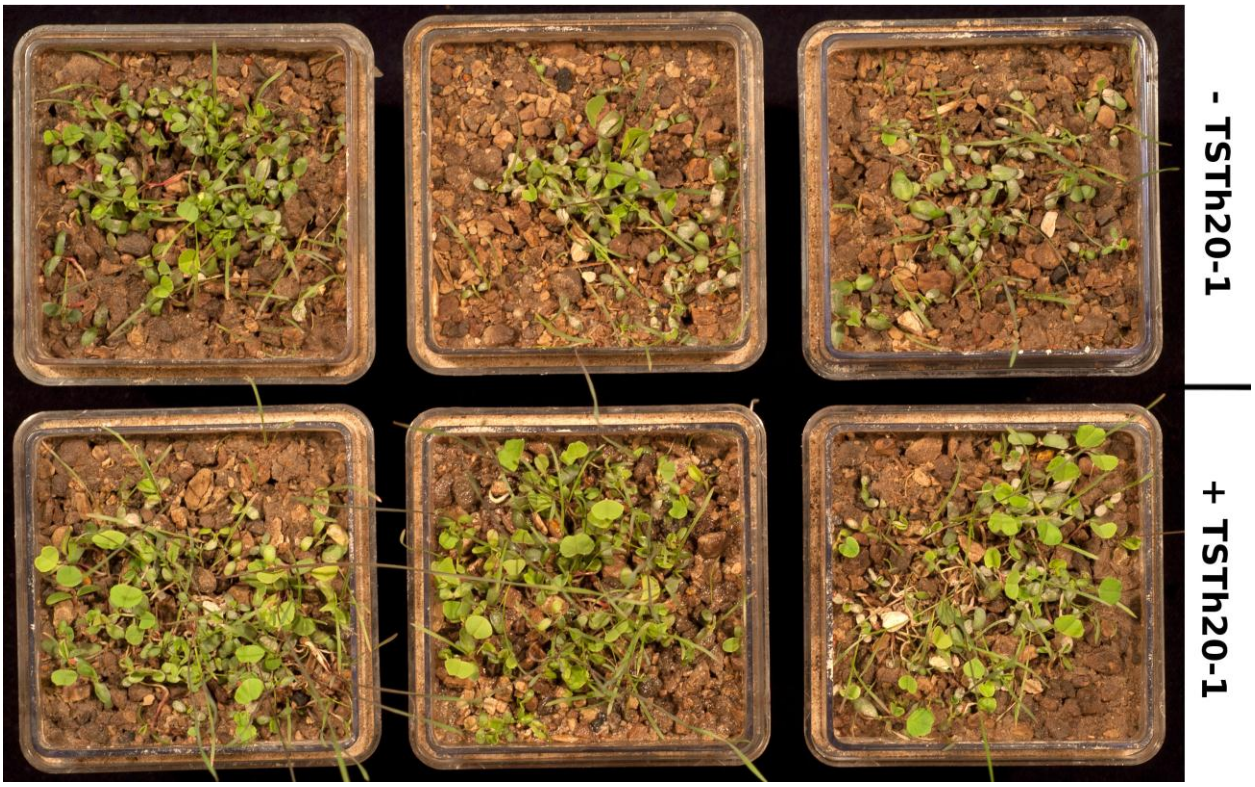

Figure 4.2. A small study investigating the growth of native species on a small volume of TS. The top row consists of native plants grown on TS in the absence of TSTh20-1 while the bottom row consists of plants grown in the presence of TSTh20-1. Qualitatively, the plants with TSTh20-1 appear both more numerous and larger, something that was not observed in experiments with larger volumes of TS.

TSTh20-1 did not appear to promote the growth of native species in meso-scale environments. The reason for this is unknown; however it may be due to the size of the rhizosphere. A small experiment was performed (Figure 4.2) where the same native species were grown in small rather than the large volumes (Magenta box vs. PVC pipe) of TS. Plants inoculated with TSTh20-1 grew significantly faster, at least in the first month of the experiment. This suggests that the volume of the soil to which the plant in exposed has an effect. One might expect plants to do better in larger than smaller soil volumes because more nutrients are accessible. A possible explanation to this might be that a toxic agent, such as NAs, present in TS. A smaller soil volume might allow the plant and fungus to more effectively degrade this toxin or

be exposed to a lower dose leading to better growth, whereas in larger soil volumes the dose of the toxin may be larger or more persistent. However, as toxins have not been studied in TS, so this must remain a hypothesis. Further studies investigating this hypothesis will allow for a better understanding to the unexpected relationship between soil volume and plant health.

4.3. The possibility of hydrocarbon degradation

In the meso-scale experiment using TS from Company A, a large spike in the amount of total hydrocarbons was observed when plants inoculated with TSTh20-1 were grown on TS. Unfortunately, the TPH analysis was performed as a single replicate consisting of an even mixture of 3 samples. This makes the results statistically insignificant. A repeat of this experiment was performed using TS from Company B, due to the limited quantities of TS available from Company A. The analysis of the Company B TS after the growth period showed no change in the hydrocarbon profile. These two companies utilize different extraction methods with different extraction efficiencies and specificities for different types of hydrocarbons. This offers a potential explanation for the reason for the increase in TPH seen in one TS but not the other.

It is known that Company A rejects asphaltenes from their extraction process (Devenny, 2009) and sends them to tailings, leaving a bank of large hydrocarbon molecules that are undetectable via CCME methods, whereas hydrocarbons present in TS from Company B do not have this potential source.

It may be that TSTh20-1 can degrade these large hydrocarbons into smaller pieces, pieces that are small enough to become visible in the CCME method (<50 carbons). In the absence of this bank of large hydrocarbons, no change in TPH was observed. This hypothesis is supported by the enhanced plant growth on smaller volumes of TS seen in the previous section, suggesting that a degradable toxin is present and is more rapidly degraded in a smaller environment, allowing for greater plant growth. However, since the results using tailings from Company A did not have proper analytical replication, and since their composition differed from Company B TS, this remains an untested hypothesis. More studies will be required to evaluate the value of

TSTh20-1 as a hydrocarbon degrading fungus, and whether this degradation correlates with enhanced plant growth.

4.4. Mechanisms of plant growth promotion

TSTh20-1 strongly solubilized Al phosphate precipitates. Moderate solubilization of Ca and Fe phosphates was observed, but solubilization was weaker than a known phosphate solubilizer. The ability to liberate nutrients in an oligotrophic environment is essential for survival of both the fungus and plant. It was not investigated whether the liberated phosphate is taken up by the plant or if it remains within the endophyte and should be the subject of a future study.

TSTh20-1 did not produce indole-type compounds or siderophores, when grown on synthetic media in the absence of a plant. However, potentially, TSTh20-1 could have induced production of either type of compound when *in planta*. Some strains of *T. harzianum* (T-22) have been shown to alter genetic expression and hormone profiles within plants. Thus it a strong possibility that the fungus does not produce these compounds itself, but rather alters the expression of the genes within the plant by other means. TSTh20-1 did not protect green plant tissue from reactive oxygen species (ROS) as other fungal endophytes have been shown to do (Marquez *et al*, 2007; Hamilton *et al*, 2012). However, the endophyte might be protecting the plant from ROS generated in other tissues. This cannot be tested using the paraquat assay as paraquat is only effective in green tissue. These preliminary results looking at the potential mechanisms that TSTh20-1 may employ to benefit the host plant in a harsh environment have proven inconclusive at best. The mechanisms that Class 2 fungal endophytes use to enhance plant growth are currently not yet clarified in the literature, leaving little base to compare these results to, or to determine if the experiments performed were adequate enough to test for these mechanisms.

4.5. Use of endophytes in reclamation may reduce costs and increase success rates

Despite our incomplete knowledge about the plant growth promoting mechanisms used by TSTh20-1, this endophyte still holds the potential to enhance current efforts to reclaim lands

disturbed by oil sands surface mining. First, TSTh20-1 has been shown to increase drought recovery in plants that have severely wilted. Second, TSTh20-1 has been shown to enhance seed germination both on potting mix and on TS. Third, TSTh20-1 enhances plant growth on TS without added mineral fertilizer. Fourth, TSTh20-1 readily colonizes a wide range of grasses, legumes and other forbs, many of which are suitable for TS reclamation strategies.

TS reclamation is a multi-stage process, at least for methods currently used by mining companies. The cost of TS reclamation has the potential to outgrow the money set aside for it. The use of TSTh20-1 in the early stages, during establishment of the perennial grass cover, is expected to reduce cost. The improved seed germination rates that TSTh20-1 would allow for industrial seeders to be used. Further, I have shown that treating TS with 1 % Tween 20 helps improve water penetration of hydrophobic TS, and so offers a potentially much higher germination success. In addition, TSTh20-1 helps plants rapidly recover from water stress situations. As a result, TSTh20-1 colonized plants will better be able to utilize small amounts of water received in hydrophobic soils before it evaporates or is lost as runoff allowing for lower death rates for plants. TSTh20-1 liberates phosphate from recalcitrant soil precipitates. Although I fully expect that oil sands companies will fertilize their sites during perennial cover establishment, lower rates of fertilization or only one fertilizer application may be required. All of these features combined will allow lower maintenance of sites under temporary reclamation and when combined with other technologies may greatly reduce the costs of reclamation.

4.6. Future directions

This thesis research project was a preliminary investigation into the suitability of TSTh20-1 for use in reclamation and remediation of sites impacted by oil sands mining activities. In addition, I explored the mechanisms potentially used by TSTh20-1 to enhance plant growth on dry, nutrient-limited, hydrocarbon-contaminated TS.

Some of the results in this study have proven to be inconclusive, largely due to the limited amount of TS available and limited information in the literature. As a result the scale and types of experiments necessary for conclusive, statistically sound, results were unable to be performed. Current results have only been able to provide intriguing and highly promising glimpses of the

potential for this research. Taken together, despite limitations, preliminary results indicate many interesting future directions for this work.

The most interesting finding of the research presented in this thesis is the possibility that TSTh20-1 is degrading large and complex hydrocarbons, however more concrete evidence is needed. These are found in certain types of tailings, including those from company A. Degradation of large hydrocarbon molecules may result in enhanced plant growth dependent on soil volume and the rate of degradation. Future studies would be best focused on tailings that contain large amounts of asphaltenes and related compounds, namely those from Company A. For statistical rigor, experiments should use larger numbers of replicates that are correlated to soil enzyme activities. Similarly, it would be very interesting to examine the concentration of naphthenic acids in TS and use established methods to determine if they are degraded when exposed to TSTh20-1.

A great increase in soil peroxidase activity was observed in TS when plants inoculated with TSTh20-1 were present, but not when TSTh20-1 alone was present. Notably, TSTh20-1 can grow in TS without need of additional fermentable carbon sources. It is possible that all peroxidase activity observed was plant derived with increased production of the enzyme being induced by the fungus. This could be studied using protein-based techniques. For example, a comparison of the type and activity of peroxidases present in the soil when TSTh20-1, the plant, and inoculated plants are present would be useful to understanding how best to apply TSTh20-1 to decontaminate as well as revegetate TS. Similarly, molecular means such as qPCR could be used to asses changes in peroxidase gene activity in plants inoculated with TSTh20-1.

Methods other than those used in this study can be used to determine the possible mechanisms employed by TSTh20-1 to enhance plant growth. Plant hormone profiles could be examined to determine if TSTh20-1 is altering the levels and types of hormones present. Siderophores could be better tested by extracting them from soil grown with plants inoculated with TSTh20-1 and without TSTh20-1 using the same CAS assay but analyzed spectrophotometrically. Finally, nutrient solubilization in soil could be evaluated by creating an

artificial soil with known amounts of insoluble nutrients. Plants grown on this artificial soil could be evaluated for nutrient content when grown with or without TSTh20-1 under these conditions to determine if TSTh20-1 is assisting in uptake of recalcitrant nutrients.

Most importantly, a future study should establish a field site to test the effectiveness of TSTh20-1 in real world conditions. A field site could be used to evaluate if TSTh20-1 promotes plant growth the same way observed in lab conditions, degrades hydrocarbons, and leads to reduced need for maintenance (and thusly cost) at the site.

5.0. REFERENCES

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