Endophytic Fungi Associated with Pioneer Plants Growing on the Athabasca Oil Sands

By

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In partial fulfillment for the Degree of Master of Science

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ABSTRACT

Fungal endophytes live inside plants without causing apparent symptoms of infection. All plant species surveyed thus far, including liverworts, mosses, seedless vascular plants, conifers, and angiosperms, harbor one or more endophytic fungi. Fungal endophytes can be divided into four groups including class 1, class 2, class 3 and class 4 endophytic fungi according to host range, colonization pattern, transmission, and ecological function. Class 2 fungal endophytes benefit their host by increasing environmental stress tolerance (i.e. water, temperature, salt) in a habitat-specific manner. In my study, class 2 fungal endophytes were studied from weedy plants growing in an environmentally stressed area: mine tailings from the Athabasca oil sands. This area is a vast hydrocarbon reserve in western Canada that supplies 10% of Canadian oil needs. Hydrocarbons are extracted from tar sands with hot water, alkali, and solvents. The tailing sands can later be remediated (by adding organic material and fertilizer) to establish new plant communities. Prior to remediation, tailing sands have extremely low content of organic carbon and available minerals, and are hydrophobic compared to unimpacted and remediated soils. Nevertheless, *Taraxacum* (dandelion) and *Sonchus* (sow-thistle) can colonize extracted tailing sands even prior to remediation. Preliminary results show that pioneer plants have similar fungal abundance as plants of unextracted treatments. Fungal endophytes were isolated from surface sterilized *Taraxacum* and *Sonchus* that had been growing upon unimpacted, remediated and extracted soil. Fungi isolated in this way included *Alternaria, Tricoderma, Fusarium* and an unidentified Perithecial Ascomycote. These endophytic fungi were used to inoculate tomato plants in a greenhouse trial to determine whether they confer stress tolerance to host plants, especially for drought and low mineral nutrition. Before exposing the tomato plants to environmental stresses, the specific endophytic fungal strains applied were successfully
recovered from tomato plants originally inoculated with the same endophytic fungi. Although the other endophytic fungi turned out to be harmful to the tomato plants in the test, a *Trichoderma* spp. strain isolated from samples of extracted treatment appears to confer tolerance of tailing sands to the tomato plants. This *Trichoderma* spp. strain which we can call TSTh20-1 was molecularly identified as *Trichoderma harzianum*. Despite an identification to species, all strains of *T. harzianum* are not necessarily identical regarding strain-specific attributes. Using similar techniques described here, it is possible to isolate and potentially use beneficial class 2 endophytic fungal strains for the remediation process in the Athabasca oil sands or to assist plant growth in other high stress environments.
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TABLE OF CONTENTS

PERMISSION TO USE .................................................................................................................................. i

ABSTRACT .................................................................................................................................................. ii

ACKNOWLEDGMENTS .............................................................................................................................. iv

TABLE OF CONTENTS ............................................................................................................................. v

LIST OF ABBREVIATIONS ....................................................................................................................... vii

LIST OF FIGURES ...................................................................................................................................... x

LIST OF TABLES ....................................................................................................................................... xii

I. INTRODUCTION ...................................................................................................................................... 1

1.1. Symbiotic relationship between plants and fungi .............................................................................. 1

1.2. Arbuscular mycorrhizal fungi (AMF) ................................................................................................. 3

1.2.1. Definition ........................................................................................................................................ 3

1.2.2. Classification .................................................................................................................................. 4

1.2.3. Ecology and Evolution ................................................................................................................... 6

1.2.4. Development of AMF symbiosis ................................................................................................... 7

1.2.5. Typical fungal structures in AMF symbiosis ............................................................................... 8

1.2.5.1. Arbuscules and hyphal coils ...................................................................................................... 8

1.2.5.2. Vesicles ...................................................................................................................................... 8

1.2.6. AMF benefits .................................................................................................................................. 9

1.2.7. Limitations for application of AMF ............................................................................................ 10

1.3. Endophytic fungi classification and host range .................................................................................. 11

1.3.1. Endophytic fungi classification and host range ............................................................................ 11

1.3.2. Development of class 2 endophyte symbiosis ........................................................................... 12

1.3.3. In vitro culture of endophytic fungi ............................................................................................. 12

1.3.4. Molecular identification of endophytic fungi ............................................................................. 13

1.3.5. Target DNA sequences ................................................................................................................. 13

1.3.6. Primer design .................................................................................................................................. 14

1.3.7. Polymerase chain reaction (PCR) ................................................................................................. 15

1.4. Class 2 endophyte fungi and plants .................................................................................................... 15

1.4.1. Endophytic fungi benefits ............................................................................................................ 16

1.4.2. Habitat-adapted symbiosis ........................................................................................................... 17

1.5. Trichoderma harzianum ....................................................................................................................... 19

1.6. Athabasca oil sands ............................................................................................................................. 19

1.6.1. Geographical and geological characteristics ................................................................................. 19

1.6.2. Oil extraction process .................................................................................................................... 21

1.6.3. Current remediation process in Athabasca oil sands ................................................................. 23

1.6.4. Soil environment of tailing sands ................................................................................................. 24

1.7. Study objectives .................................................................................................................................. 24

1.7.1. What fungi are present in *Taraxacum* and *Sonchus* plant species? ........................................ 24

1.7.2. What type of fungi is more practical in assisting the remediation process? ................................. 25

1.7.3. Can endophyte fungi confer tolerance to stress on tailing sands to tomato plants? .................. 25

1.7.4. Endophyte species characterization ............................................................................................ 26
II. MATERIALS AND METHODS ...................................................................................................... 26
  2.1. Sample design ...................................................................................................................... 26
  2.2. Measurement of soil pH .................................................................................................... 27
  2.2.1. Measurement of soil organic carbon ................................................................................ 28
  2.2.2. Measurement of soil K, Ca, Mg, Na ................................................................................ 29
  2.2.3. Measurement of soil available N .................................................................................. 30
  2.2.4. Measurement of soil extractable P ............................................................................... 30
  2.2.5. Measurement of soil hydrophobicity ........................................................................... 31
  2.2.6. Soil hydrophobicity ....................................................................................................... 31
  2.3. Detection of AMF and endophytic fungi in plant tissue by wide field epifluorescence microscope and CLSM ......................................................................................... 31
  2.4. Analysis of fungal abundance ......................................................................................... 34
  2.5. Purification and morphological identification of endophytic fungi strains ......................... 35
  2.6. Plant colonization ............................................................................................................. 36
  2.7. Abiotic stresses assay ....................................................................................................... 37
  2.8. Reisolation and identification of endophytic fungi from inoculated plant tissue ................. 38
  2.9. DNA extraction ................................................................................................................ 38
  2.10. Polymerase chain reaction and sequence ....................................................................... 39
III. RESULTS .................................................................................................................................... 41
  3.1. Soil assay ........................................................................................................................... 41
  3.2. Endorhizal fungi structures observed and relative abundance in dandelion (Taraxacum officinale) samples from Athabasca oil sands ........................................................................... 43
  3.3. Recovery and morphological identification of endophytic fungi from Taraxacum and Sonchus samples ....................................................................................................................... 47
  3.4. Detection of endophytic fungi inside of plant tissue ......................................................... 52
  3.5. Re-isolating inoculated endophytic fungi from tomato plants ......................................... 53
IV. DISCUSSION ............................................................................................................................. 55
  4.1. Relationship between endorhizal fungal abundance and soil environment ....................... 55
  4.2. Limitation of methods involved in endophytic fungal recovery and morphological identification ................................................................................................................................. 62
  4.3. Possible mechanisms of habitat-specific stress tolerance to host plants conferred by class 2 endophytic fungi .............................................................................................................. 63
  4.4. Molecular identification of Trichoderma strain isolated from tailing sands ......................... 66
  4.5. Future research directions .................................................................................................. 67
V. APPENDIX ................................................................................................................................. 69
VI. REFERENCES .......................................................................................................................... 70
LIST OF ABBREVIATIONS

ABA  Abscisic Acid
act  Actin
AFLP  Amplified fragment length polymorphism
AM  Arbuscular mycorrhiza
AMF  Arbuscular mycorrhizal fungi
ANOVA  Analysis of variance
B  Boron
BLAST  Basic local alignment search tool
Ca  Calcium
CaCl₂  Calcium chloride
cal  Calmodulin
CLSM  Confocal laser scanning microscopy
CO₂  Carbon dioxide
Cu  Copper
dATP  Deoxyadenosine triphosphate
dCTP  Deoxycytidine triphosphate
ddH₂O  Double distilled water
dGTP  Deoxyguanosine triphosphate
DNA  Deoxyribonucleic acid
DSE  Dark septate endophyte
dTTP  Deoxythymidine triphosphate
EDTA  Ethylenediaminetetraacetic acid
<table>
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<td>Elongation factor gene</td>
</tr>
<tr>
<td>Fe</td>
<td>Iron</td>
</tr>
<tr>
<td>FE</td>
<td>Fine endophyte</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared</td>
</tr>
<tr>
<td>ICP-AES</td>
<td>Inductively coupled plasma atomic emission spectroscopy</td>
</tr>
<tr>
<td>ITS</td>
<td>Internal transcribed spacer</td>
</tr>
<tr>
<td>K</td>
<td>Potassium</td>
</tr>
<tr>
<td>K₂O</td>
<td>Potash</td>
</tr>
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<td>Potassium chloride</td>
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<tr>
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</tr>
<tr>
<td>MQM</td>
<td>Multiple quantitation method</td>
</tr>
<tr>
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<td>Nitrogen</td>
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<td>(NH₄)₂SO₄</td>
<td>Ammonium sulfate</td>
</tr>
<tr>
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<td>Optical density</td>
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<td>P</td>
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</table>
PAH  Polycyclic aromatic hydrocarbon
PCR  Polymerase chain reaction
PDA  Potato dextrose agar
P₂O₅  Phosphoric acid
ppm  Parts per million
PVAG  Polyvinyl alcohol
RAPD  Random amplification of polymorphic DNA
rDNA  Ribosomal DNA
RNA  Ribonucleic acid
ROS  Reactive oxygen species
rpm  Rotations per minute
rRNA  Ribosomal RNA
S  Sulfur
SE  Septate endophyte
spp  Species
SSU  Small subunit
Tris-HCl  Tris (hydroxymethyl) aminomethane-hydrochloric acid
T-22  Trichoderma harzianum Rifai 1295-22
UV  Ultra violet
Zn  Zinc
LIST OF FIGURES

Figure 1.2.2.  Phylogenetic tree of fungus showing division of Glomeromycota (AMF and relatives)…………………………………………………………………………………5

Figure 1.3.5.1.  The amplification sites of universal primers……………………………………14

Figure 1.3.5.2.  EF-1α gene structure and location of primers for amplification…………14

Figure 1.6.1.  Map of Canada highlighting the oil sands located in Alberta…………………20

Figure 2.1.  Photos of the three different treatments taken from the Athabasca oil sands: unimpacted, extracted and remediated………………………………………27

Figure 2.3.  Lateral roots of dandelion (Taraxacum officinale)…………………………….32

Figure 2.4.1.  The scanning procedure for the Multiple Quantitation Method………………34

Figure 3.1.1.  Hydrophobicity assay showing soil samples of unimpacted, extracted and remediated treatments…………………………………………………………43

Figure 3.2.1.  AMF and FE endorizal fungi in lactofuchsin-stained dandelion lateral roots from tailing sands viewed by CLSM………………………………………45

Figure 3.2.2.  SE and DSE fungi in lactofuchsin-stained dandelion lateral roots from tailing sands viewed by CLSM……………………………………………………46

Figure 3.3.1.  Fungal endophyte represented in Taraxacum and Sonchus samples of extracted treatment……………………………………………………………48

Figure 3.3.2.  Endophytic fungi isolated from Taraxacum and Sonchus showing characteristic spore morphologies………………………………………49

Figure 3.4.1.  Photographs of axenic and Trichoderma spp. inoculated tomato seedlings in potting mixture grown for 2 weeks……………………………………….50
Figure 3.4.2.  Photographs of axenic and *Trichoderma* spp. inoculated tomato plants after they grew on tailing sands for 2 weeks……………………………..51

Figure 3.5.1.  Confocal microscope pictures of fungi associated with roots of rice seedlings inoculated with *Fusarium* and *Curvularia*……………………..53

Figure 3.6.1.   Pictures of fungi that had recovered from axenic plants, and plants inoculated with *Fusarium* spp, *Trichoderma* spp., and *Alternaria* spp………………………………………………………………………..54

Figure 3.7.1.  PCR results………………………………………………………………55
LIST OF TABLES

Table 2.11. Primers used in PCR………………………………………………………..41

Table 3.1.1 Soil test data obtained from unimpacted, extracted, and remediated treatments ………………………………………………………………..42

Table 3.2.1. Endorhizal fungal colonization of dandelion roots subjected to different treatments ………………………………………………………………..47

Table 3.4.1. Weight of axenic plants and plants inoculated with Trichoderma after two weeks growth on tailing sands…………………………………51

Table 3.4.2. Comparison of moisture content in drought tolerance experiment………52
I. INTRODUCTION

1.1. Symbiotic relationship between plants and fungi

Symbiosis is a mutually beneficial interaction between two organisms. Most plants including liverworts, mosses, seedfree vascular plants, conifers, and angiosperms are symbiotic with fungi (Rodriguez et al., 2004; Arnold and Lutzoni, 2007) and symbiotic fungi play important roles in the structure, function, and health of plant communities (Petrini, 1986; Bacon and Hill, 1996; Rodriguez and Redman, 1997; Clay and Holah, 1999; Read, 1999). There are two major groups of fungal symbionts associated with plants: mycorrhizal fungi that reside not only in roots but also extend out into the rhizosphere; and fungal endophytes that reside entirely within plant tissues and may be associated with roots, stems and/or leaves. Both types of interaction are extremely ancient, having been documented in Early Devonian fossils from the Rhynie chert (Krings et al., 2007).

Arbuscular mycorrhizal fungi (AMF) have aseptate hyphae, produce characteristic intracellular structures (arbuscule or coil) within roots, are transmitted by means of asexual spores in the soil, and are obligate biotrophs. The latter means that their growth requires another living organism. For symbiosis between AMF and terrestrial plants, AMF receive shelter and photosynthetic carbohydrates from the host plants while the host plants receive mineral nutrients from AMF (Simon, 1996). AMF are also known for enhancing host plant tolerance to water deficit (Koske and Gemma, 1997; Augé, 2001), high heavy metal concentration (Schutzendubel and Polle, 2002; Goehre and Paszkowski, 2006), some kinds of soil-borne pathogens and toxicants in the soil (Norman et al., 1996; Leung et al., 2007).

Septate endophytes (SE) have septate hyphae and colonization of host plants, via sporulation (generation of spores), occurs by vertically and horizontally transmitting spores. Vertical
transmission of SE spores is associated with host plant senescence and spores are transmitted to
the next plant generation via the seed coat, and rhizomes (Rodriguez et al., 2008a). SE spores
can also be horizontally transmitted to nearby plants by means of rain, wind, or direct contact
(Rodriguez et al., 2008a). Similar to AMF, SE fungi gain shelter and nutrients from host plants.
Various functional classes of SE are known to provide benefits to the host plant including
reduced animal herbivory and assist host plants to adapt to high-stress environments (Wilson,
1995; Rodriguez et al., 2004; Lehtonen et al., 2005) as well as improve their host’s competitive
ability (Clay et al., 1993).

Relative to the amount of research about the symbiotic relationship between AMF and
terrestrial plants, much less work has focused on how the endophyte fungal symbiont promotes
the growth of host plants. Porter et al (1979) described early research by Robbins and Bacon
(1976) where endophytic fungi were described in grasses (such as tall fescue, Festuca
arundinacea) growing on a pasture, however, the function of endophytic fungi (such as
Neotyphodium spp.) was not yet clear. It is now known that cool season grasses infected by the
fungal endophyte Neotyphodium spp. are more vigorous, more toxic to herbivores, and more
resistant to drought compared to uninfected cold season grasses (Rice et al., 1990; Clay and
Holah, 1999; Malinowski and Belesky, 2000).

Four classes of endophytic fungi have now been described (Rodriguez et al., 2008a), each of
which are different in their host range, colonization pattern, transmission, and ecological
function (Rodriguez and Redman, 2008). As a typical example of class 1 endophytes, Clay and
Schardl (2002) suggested that the grass fungal endophytes which are found in cold-seasoned
grass may constitute a monophyletic clade with the fungal family Clavicipitaceae, whereas the
other 3 classes were known as non-Clavicipitaceous. Both mycorrhizae and Clavicipitaceae
endophytes have been well studied, while less is known about non-Clavicipitaceae (class 2-4) endophytes, which may represent a relatively large group of fungal symbionts, particularly class 2 (Rodriguez and Redman, 2004; Rodriguez et al., 2008a).

The Rodriguez group’s research has focused on class 2 endophytic fungi that have a wide range of host plants, implying a vast potential for application. Although class 2 endophyte fungi have not been as extensively studied, their lab has already provided evidence to show that class 2 endophyte fungi confer the tolerance of host plants to extreme environments including high temperature, drought and high salt content (Redman et al., 2002; Rodriguez et al., 2004; Rodriguez and Redman, 2007). A fungus strain in *Curvularia protuberata* was shown to assist host plants to adapt to geothermal soil (Redman et al., 2002), a *Colletotrichum magna* strain conferred the tolerance of host plants to drought (Redman et al., 2001) and *Fusarium culmorum* has been shown to confer salt tolerance to the host plant (Rodriguez et al., 2008b). Fungal endophytes not only promote stress-tolerance, but also increase nutrient acquisition and growth rates of their host (Rodriguez et al., 2004).

1.2. **Arbuscular mycorrhizal fungi (AMF)**

1.2.1. **Definition**

Arbuscular mycorrhizal fungi (AMF) are a group of common soil-borne fungi that are associated with the roots of over 80% of all terrestrial plants. As the most widespread type of mycorrhiza, this symbiosis is highly significant on a global scale. Although AMF are obligate biotrophs, the arbuscular mycorrhiza association is a symbiosis that benefits both partners. The fungal mycelium absorbs mineral nutrients from soil, including N, P, K, Ca, S, Fe, Mn, Cu, and Zn (Garg et al., 2006), which are necessary for the growth of host plants (Smith and Read, 1997;
A commonly accepted opinion is that increased phosphate uptake in plants colonized by AMF depends mainly on the external hyphae that grow into the soil outside of the root’s P depletion zone which develops around the root. Growing roots generally deplete the rhizosphere of soluble phosphate more quickly than replenishment can take place (Kraus et al., 1987; Tawaraya et al., 2006).

1.2.2. Classification

AMF were previously placed in the class Glomeromycetes within the division Zygomycota based on the morphological similarities, for example, the interpretation of AMF spores as azygospores, which are zygospores with only one gametangium (Walker, 1983; Morton and Benny, 1990). Currently, AMF are placed into their own phylum, the Glomeromycota, based on molecular evidence of the small subunit (SSU) ribosomal RNA (rRNA) gene (Figure 1.2.2) (Schussler et al., 2001). AMF were identified to genus based on methods which focused on the morphology of the asexual spore. Spore wall structure (i.e. color, ornamentation pattern, thickness, histochemical reactivity) and spore development offer most of the information to identify AMF in this method (Bentivenga and Morton, 1994; Franke and Morton, 1994).

Obtaining AMF spores from environmental samples can be inconsistent due to variable spore number, parasitization of spores, spore viability, environmental alteration of spores such as discoloration and season-dependent spore density, which can lead to inaccurate identification (Bever et al., 2001). Since AMF are obligate biotrophs, they cannot be cultured in the absence of host plant roots (Bago and Bacard, 2002) so highly limited sources of obtaining AMF spores severely hampers large scale experiments using AMF. In contrast to the limitations of classical morphological identification techniques, using molecular methods in AMF identification is
extremely valuable. Typically, the nucleic information derived from the AMF is from ribosomal DNA (rDNA) (Redecker, 2000a). The internal transcribed spacer (ITS) region comprises highly conserved functional sequences and more variable spacers that are not under selective pressure. ITS regions are used for the identification or phylogenetic analyses of a wide range of fungi and fungus-like organisms (Redecker et al., 1997; Bever et al., 2001). Redecker (2000a) designed group-specific primers to amplify the ITS and 18 S rRNA gene fragments for identifying major subgroups of AMF. These group-specific primers have been widely used and proved to be effective for identifying AMF collected in field (Redecker et al., 2003; Wubet et al., 2003; Hijri et al., 2006; Shepherd et al., 2007). Several new genera and families have been erected recently (Walker and Schussler, 2004; Walker et al., 2007) and AMF species are placed under seven genera: Acaulospora, Entrophospora, Archaeospora, Glomus, Paraglomus, Gigaspora and Scutellospora (Khade, 2008).

Figure 1.2.2. Phylogenetic tree of fungus (left) showing division of Glomeromycota (AMF and relatives). Based on work from Schussler et al., (2001) and Khade (2008).
1.2.3. Ecology and Evolution

Early ancestors of modern AMF have been found in Rhynie chert fossils formed in Early Devonian (410 million years ago) (Strullu-Derrien and Strullu, 2007), which show that the rhizoids of *Nothia* have fungal colonization and the hyphae of some mutualistic colonists were encased by plant cell walls (Berbee and Taylor, 2007). Although not showing an association with plant tissue, the earliest fossil evidence of AMF, represented as entangled, occasionally branching, nonseptate hyphae and spores, were found in rocks over 460-455 million years old (Redecker *et al*., 2000b). All of the spores in the fossils retain their subtending hyphae which have a diameter of 3-5 μm. The spore wall consists of a single layer. According to their shape, size, and hyphal connection, the spores are very similar to those of modern fungi in the genus *Glomus*. Also, the loose spore clusters that were found in the fossils are frequently formed by AMF but are unknown from algae, other fungi, or Oomycetes (Redecker *et al*., 2000b). This evidence implies that the fungi found in the fossils strongly resemble modern AMF.

Fossil evidence strongly suggests that major groups of fungi were present even before the first terrestrial vascular plants that arose in the Silurian period (444-417 million years ago) (Redecker *et al*., 2000b). In terms of time, this supports the hypothesis that AMF association may have assisted ancestral vascular plants to invade land (Pirozynski and Malloch, 1975). During the Silurian period, it is proposed that ancestors of modern plants lacked the ability of extracting essential nutrients from the soil or perhaps sufficient quantities. Since the ancestors of modern AMF did not have the ability of producing carbohydrates (Pirozynski and Malloch, 1975), the relationship was established between these two so that both of the partners could adapt to the terrestrial habitat.
1.2.4. Development of AMF symbiosis

In the modern day, the sequence of steps leading to the establishment of a functional symbiosis between AMF and host plants involves: (I) the presymbiotic phase; (II) contact and entrance of the fungus into root tissue; (III) intraradical fungal proliferation; and (IV) cell invagination and nutrient transfer (Paszkowski, 2006). In the presymbiotic stage, the fungus senses a host-derived signal (such as strigolactones) leading to intensified hyphal branching that is possibly to increase the chance of contact with a host root (Akiyama et al., 2005). The concentration of strigolactone in root exudates coincides with the host specificity of AMF so that the host plant can be recognized. For example, the non-mycotrophic plant *Arabidopsis thaliana* produces very low amounts of strigolactones compared to hosts of AMF (i.e. carrot and tobacco) (Westwood, 2000). After host plant recognition, AMF hyphae penetrate root hair cell or epidermal cells through exerting a hydrostatic pressure at the hyphal tip as well as by producing cell wall degrading enzymes to get through the localized cell wall (Bonfante and Perotto, 1995). For example, *Glomus mosseae* showed higher activities of cell wall degrading enzymes such as pectinase in AMF colonized roots compared to non-symbiotic roots (Garcia-Romera et al., 1991; Garcia-Garrido et al., 1992). After AMF successfully penetrate into the host roots, their aseptate hyphae grow through the cortex in both directions from the entry point. The host response to intracellular hyphal invasion such as the formation of arbuscules or hyphal coils is a forming of a host-derived membrane that is continuous with the plant cell membrane (Bonfante and Perotto, 1995).
1.2.5. Typical fungal structures in AMF symbiosis

AMF produce characteristic intracellular structures within roots that are readily distinguished with confocal laser scanning microscopy (CLSM). Arbuscules and/or hyphal coils are observed in AMF infected plant roots. Vesicles are also a typical AMF structure found in AMF infected plants.

1.2.5.1. Arbuscules and hyphal coils

The highly branched tree-like fungal structures which are formed by repeated branching of intracellular hyphae within root cortical cells were named ‘arbuscules’ by Gallaud (1905). Hyphal loops inside of plant cell are called hyphal coils which are frequently found growing in the outer cortex (Cavagnaro et al., 2001). The formation of an arbuscule starts from a trunk hypha (5-10 μm in diameter) proliferating fine branch hyphae (<1 μm diameter). The formation of arbuscules is typically accompanied with plant vacuole fragmentation and the movement of the nucleus to the center of the cell (Bonfante and Perotto, 1995). One of the significant functions of arbuscules and hyphal coils is to increase the contact area between the fungus and cortical cells of the root. The arbuscules and hyphal coils are considered to be the structures responsible for the exchange of carbohydrates and mineral nutrients between AMF and host plants (Smith and Smith, 1990).

1.2.5.2. Vesicles

Vesicles are not present in every AMF symbiosis and it is thought that some AMF species don’t produce them at all (Smith and Smith, 1997). Vesicles are hyphal swellings in the root
cortex that contain lipids and nuclei (Smith and Smith, 1997). Vesicles have the function of storage and when they are old, they also play the role of reproduction (Miyasaka and Habte, 2001). Biermann and Linderman (1983) successfully inoculated previously uninfected roots using vesicles of some species of AMF as propagules. Some research shows vesicles start to grow soon after the arbuscules are formed and continue to develop when the arbuscules degenerate (Peterson et al., 2004).

1.2.6. AMF benefits

AMF benefits to host plants are related to assisting the host plants to increase the uptake of mineral nutrients, especially phosphorus (P). P is an important plant macronutrient building molecule, because it is necessary for producing nucleic acids and phospholipids, and for regulating enzymatic and metabolic reactions (Theodorou and Plaxton, 1993). Compared to plant roots, AMF are much more efficient in capturing P (Bolan, 1991). Cox and Tinker (1976) measured the quantity of P transferred per unit time then divided it by the area of the interface (measuring mosaics of electron micrographs from fixed transverse sections) to estimate the P flux at 13 nmol/m²/s¹ along the hyphae in mycorrhizae. This P transfer speed is up to six times faster than the P transfer speed in the root hairs. Additionally, the extraradical hyphae grown into the surrounding soil increase the surface area of soil contact which also leads to more effective uptake of P. AMF may also improve soil phosphorus availability through solubilizing inorganic forms of phosphorus and mineralization of organic phosphorus (Hamel, 2004; Tawaraya et al., 2006).
1.2.7. Limitations for application of AMF

AMF are obligate biotrophs, that is, they cannot be cultured without an intimate relationship with living host plant roots and the single-spore pure culture cannot be developed. The obligate characteristic of AMF makes it extremely difficult for selecting and identifying highly effective AMF strains that have the potential of being used for either enhancing the growth of host plants or assisting environmental remediation. This obligate nature also limits the amount of spores that can be produced in lab which severely hamper the large scale application of AMF. Another limitation for the application of specific AMF association for remediation is that the beneficial effects depend on not only the combined efficacy of plants and fungi involved but also on their ecological interactions within the system (soil, water, sediment, etc.) which makes the mechanism even more complicated and more factors need to be considered in the application procedure of AMF (Kapoor et al., 2008).

1.3. Endophyte fungi

1.3.1. Definition

In contrast to AMF that only colonize in the host root, fungal endophytes reside within roots, stems and/or leaves and emerge during host senescence (Saikkonen et al., 1998). Endophyte fungi are considered as plant mutualists and live asymptomatically within the host tissues, receiving protection and nutrients, while host plants may benefit from increased resistance to herbivores, pathogens, and various abiotic stresses as well as enhanced competitive abilities (Wilson, 1995; Rodriguez and Redman, 1997; Lehtonen et al., 2005).
1.3.2. Endophytic fungi classification and host range

Endophytic fungi consist of a phylogenetically diverse group that are members of the dikaryomycota (Carroll, 1988; Scharld et al., 2004; Van Bael et al., 2005; Girlanda et al., 2006; Arnold and Lutzoni, 2007). While most endophyte fungi belong to the Ascomycota, a few belong to the Basidiomycota (Rodriguez et al., 2008a). Fungal endophytes can also be discriminated into different functional groups similar to what has been done with mycorrhizal fungi (Brundrett, 2006). Currently, endophytes are subdivided into 4 classes including class 1, class 2, class 3 and class 4 according to host range, colonization pattern, transmission, and ecological function (Rodriguez and Redman, 2008).

Class 1 endophytes are a relatively small number of species that are limited to a few monocot hosts and class 2-4 endophytes are a large number of species with broad host ranges, including both monocots and dicots (Rodriguez and Redman, 2004). Class 2 endophytes can grow in both above and below ground tissues and form extensive tissue colonization, while class 3 endophytes are restricted to above ground tissues and form highly localized infections and class 4 endophytes are restricted to roots (Rodriguez et al., 2008a). Only class 2 endophytes have been shown to confer habitat-adapted stress tolerance, although most class 3 and 4 endophytes have not been assessed for conferring fitness benefits to hosts (Rodriguez et al., 2008a). Class 2 fungal endophytes not only have a broad host range, but have been shown to confer the adaptation of host plants to high-stressed environments and are typically of high infection frequencies in plants growing in high stress habitats (Redman et al., 1999a, 2001, 2002; Rodriguez and Redman, 2007). Based on these qualities, I hypothesized that class 2 endophytic fungi may assist plant growth in the Athabasca tailing sands (a high abiotic stress environment).
Abiotic stress is the negative impact of physical and chemical factors (i.e. temperature, rainfall, pH, oxygen content, etc.) on living organisms in a specific environments, which is essentially unavoidable and naturally occurring. The Athabasca tailing sands are a high-stress environment that limits plant growth due to major abiotic stress factors such as hydrocarbon residues, alkali that is widely used in the oil extraction process, and extremely low mineral nutrient content.

1.3.3. Development of class 2 endophyte symbiosis

Similar to other endophytes, class 2 endophytic fungi colonize plants by using hyphae to penetrate into the plant tissue or through infection structures such as appressoria (Ernst et al., 2003). The growth of the fungi through the plant tissues in primarily intercellular with little to no injury on host cells (Rodriguez et al., 2008a). Sporulation occurs rapidly by means of emergence during host senescence (Weber et al., 2004).

1.3.4. In vitro culture of endophytic fungi

Unlike AMF, which cannot be cultured on an artificial medium, endophytic fungi can be isolated and easily cultured from plant tissue on artificial medium. In addition, endophyte fungi reside entirely within plant tissues or associated with roots, stems and/or leaves, so endophyte fungi can be isolated from different parts of the plant on culture medium plates.
1.3.5. Molecular identification of endophytic fungi

1.3.5.1. Target DNA sequences

The internal transcribed spacer (ITS) region of fungal rRNA genes has been identified by previous studies as suitable targets for molecular analysis of fungal identification (Bridge and Spooner, 2001; Gardes and Bruns, 1993). ITS regions are stretches of DNA that are located between the 18S and 5.8S ribosomal DNA (rDNA) coding regions (ITS1) and between the 5.8S and 28S rDNA coding regions (ITS2) (Bakker et al., 1992) (see figure 1.3.5.1). The ITS region has a high degree of variation between species because of the relatively low evolutionary pressure acting on these non-functional sequences. Their highly varied sequence relative to the conserved flanking rRNA genes in fungi and the high copy number of rRNA genes makes it to be easy to amplify even from small quantities of DNA (Buchan et al., 2002). In my project, ITS sequence data were used as the complement of morphological methods to identify the endophyte fungi strains at genus level.

To identify the *Trichoderma* strain to species level, sequence information of an approximately 0.65 kb fragment of the protein coding translation elongation factor gene (EF-1α) (Figure 1.3.5.2), 0.5 kb section calmodulin gene (cal), 0.75 kb actin gene (act), and ITS can be combined together to provide a much greater resolution of the representatives of *Trichoderma* spp. compared to what ITS sequence alone supplies (Samuels et al., 2006). This method makes it possible to identify the *Trichoderma* strain to species level accurately.
1.3.5.1. The amplification sites of universal primers (not to scale).

1.3.5.2. Primer design

The primer pair ITS4 and ITS5/ITS1 is universal primer pair used for the amplification of the ITS1-5.8S-ITS2 target sequence (White et al., 1990) (Figure 1.3.4.1). O’Donnell et al. (1998) designed primer pair EF-1 and EF-2 to amplify translation elongation factor coding region (EF-1α) from Fusarium spp. Then primer pair EF1-728F and TEF1 rev was applied in amplification of EF-1α coding region in Trichoderma spp and showed positive results (Samuels et al., 2002). CAL-228F and CAL-737R primer pair were designed to amplify calmodulin gene.
in filamentous fungi by Carbone and Kohn in 1999, then used in identification of *Trichoderma spp* by Chaverri *et al.* (2003b). Tact1 and Tact2 primer pair were designed and successfully used for amplifying actin gene in *Trichoderma spp* by Samuels *et al.* (2006).

### 1.3.5.3. Polymerase chain reaction (PCR)

PCR is a technique widely used in molecular biology for its efficiency in amplifying the target sequence from small quantities of DNA and RNA templates (Van Tuinen *et al.*, 1998; Schwarzott *et al.*, 2001). The PCR procedure consists of four steps: 1) Denaturation 2) Annealing 3) Elongation and 4) Final elongation is performed to ensure any remaining single-stranded DNA is fully extended (Sambrook and Russell, 2001). Almost all PCR applications involve a heat-stable DNA polymerase, such as thermal *Taq* polymerase (an enzyme originally isolated from the thermophilic bacterium *Thermus aquaticus*), which assembles a new DNA strand from the nucleotides by using single-stranded DNA as a template and DNA primers as initiation.

### 1.4. Class 2 endophyte fungi and plants

Class 2 endophytes are different from other classes of endophyte fungi as they can form extensive colonization in roots, stems and leaves, are vertically transmitted by means of seed coats and/or rhizomes as well as horizontally transmitted by rain or air, and have a low abundance in the rhizosphere (Rodriguez *et al.*, 2008a). Class 2 endophyte fungi are also unique for enhancing habitat-specific stress tolerance of host plants (Redman *et al.*, 2002; Rodriguez *et
al., 2004; Rodriguez and Redman, 2007) and have high colonization rates (90-100 %) in plants growing in high stress habitats (Rodriguez et al., 2008a).

According to the relationship and mechanism between endophyte fungi and their host plants in high stress habitats, Rodriguez et al., (2008b) developed the theory of habitat-adapted symbiosis which suggests that endophyte fungi confer specific fitness benefits to host plants growing in harsh environments. The tailing sands are not optimal for plant growth because of low mineral nutrient content, low organic C, high pH, and residual hydrocarbon (Bois et al., 2006). All of these abiotic stress factors imply that a habitat-adapted symbiosis between class 2 endophytic fungi and pioneer plants can be established in Athabasca tailing sands.

1.4.1. Endophytic fungi benefits

Cool-season grass infected by class 1 endophyte fungi show better tolerance to biotic and abiotic stresses and become more competitive than non-infected grass (Marks et al., 1991; Bacon and Hill, 1996; Hill et al., 1998). Neotyphodium spp. endophytes produce a range of alkaloids (Bush et al., 1997; Leuchtmann et al., 2000) and/or stimulate the host plants to produce alkaloids and other kinds of secondary metabolites (Bush et al., 1997; Ju et al., 1998) so that the host plants can be protected from being eaten by herbivores. Bacon (1993) found that the endophyte-fungal infected grass show tolerance to water stress through early shedding of older leaves and rolling of younger leaves in addition to low stomatal conductances and development of an enhanced osmoregulatory system.

Depending on the particular extreme environment from which they were isolated, class 2 endophyte fungi may confer benefits to their host plants through: 1) drought tolerance (Redman et al., 2001); 2) salt tolerance (Rodriguez et al., 2008b); 3) heat tolerance (Redman et al., 2002);
4) pathogen resistance (Redman et al., 1999a). Rodriguez et al., (2008b) have shown that class 2 endophyte fungi isolated from agricultural, coastal and geothermal habitats confer drought tolerance and more effective water utility of their host plants. A dunegrass (*Leymus mollis*) cannot survive in coastal habitats without colonization by a strain of the habitat-adapted class 2 endophyte fungus *Fusarium culmorum* that confers salt tolerance to the host plant (Rodriguez et al., 2008b). Redman et al., (2002) compared heat tolerance of infected and non-infected *Dichanthelium lanuginosum* (tropical panic grass) plants with a strain of the class 2 endophyte fungus *Curvularia protuberata*. The symbiont and host plant were able to tolerate daily root temperature regimes of 65 °C for 10 h, followed by 37 °C for 14 h or sustained 50 °C root temperatures (Redman et al. 2002), but the non-symbiotic plants had a maximum growth temperature of 40 °C (Redman et al. 2002) as did the fungus. A particular strain of *Colletotrichum magna*, another class 2 endophyte fungus, has been shown to be able to protect plants from pathogens such as *Colletotrichum*, *Fusarium*, and *Phytophthora* (Freeman and Rodriguez, 1993; Redman et al., 2001). Although the mechanism by which class 2 endophytic fungi benefit host plants in high-stressed environments is still unknown (Rodriguez et al., 2005), rapid activation of host stress response systems after symbiotic plants are exposed to stress (Redman et al., 1999b) and synthesis of anti-stress biochemicals by the fungus (Bacon and Hill, 1996) might be involved in the adaptation process.

### 1.4.2. Habitat-adapted symbiosis

Rodriguez et al. (2008b) suggested that endophyte fungi confer specific fitness benefits related to the habitat. Without these endophyte fungi, it can be impossible for the host plants to handle habitat-imposed abiotic stresses such as drought, high temperature and salt stress.
(Rodriguez and Redman, 2008). For example, *Curvularia protuberata* can confer heat tolerance to host plants growing in Yellowstone National Park (Redman *et al.*, 2002); field and laboratory assays have shown the non-symbiotic plants either shriveled or died when the root zones are heated up to 65 °C, whereas plants infected by *C. protuberata* survived. Once the fungi and the host plant grow separately, neither of them is able to tolerate temperatures above 40 °C (Redman *et al.*, 2002). In addition, CThTV, a strain of virus that provides biochemical changes to the fungus, assists the fungus to confer heat tolerance to its host plant (Marquez *et al.*, 2007). The symbiosis relationship in this case is actually a unique three-way symbiosis and possible contributions of viruses to other endosymbionts have not yet been investigated (Redman *et al.*, 2002). Another example of habitat-specific fungal adaptation is a native dunegrass (*Leymus mollis*) on coastal beaches of Puget Sound, WA which is colonized by a fungal endophyte (*Fusarium culmorum*). A field trial showed that *L. mollis* infected by *F. culmorum* strain isolated from a coastal habitat adapted to a high salt content while *L. mollis* infected by *F. culmorum* derived from a non-coastal plant habitat could not survive there (Rodriguez *et al.*, 2008b). This fact revealed that the ability to confer salt tolerance was specific to isolates from the coastal plants, indicating that the ability to confer salt tolerance is a habitat-adapted phenomenon (Rodriguez *et al.*, 2008b)

Further evidence of habitat-specific adaptation are shown by some endophyte fungi isolated from geothermal plants confer a heat tolerance but not disease or salt tolerance and endophyte fungi which were isolated from coastal plants confer stress tolerance to salt but not heat or disease tolerance (Rodriguez *et al.*, 2008b).
1.5. *Trichoderma harzianum*

Strains of the class 2 endophyte fungus *Trichoderma harzianum* are known to control soil-borne disease, are widely used as a biocontrol agent, and also used for industrial production of cell wall degrading enzymes (Naseby *et al*., 2000). *T. harzianum* produces an enzyme that catalyzes the breakdown of chitin, a primary component of fungal cell walls (Zeilinger *et al*., 1999), so that *T. harzianum* can successfully penetrate into the cell wall of other fungi. Penetration is followed by the release of antibiotics. The antibiotics permeate the perforated hyphae and prevent resynthesis of the host cell wall (Lorito *et al*., 1996). *T. harzianum* then cause dissolution of the cytoplasm and grow within the empty host hyphae (Inbar *et al*., 1996). *T. harzianum* has also been shown to induce metabolic changes in plants that increase the resistance of to a wide range of plant-pathogenic microorganisms and viruses (Harman *et al*., 2004). A *T. harzianum* strain, T-22 has been shown to increase the growth of plant and root development as well as solubilize various plant nutrients, such as rock phosphate, Fe$^{3+}$, Cu$^{2+}$, Mn$^{4+}$ and Zn$^0$, that can be unavailable to plants in certain soils (Altomare *et al*., 1999), so *T. harzianum* is also considered to have evolved as an opportunistic plant symbiont (Harman *et al*., 2004).

1.6. Athabasca oil sands

1.6.1. Geographical and geological characteristics

The Athabasca oil sands in Alberta, Canada, provide an abundant source of crude oil. Although there are three large oil sand development regions in Alberta (Figure 1.6.1), the Athabasca oil sands are the largest in the world and account for $>95\%$ of the in-place resources of bitumen in North America (Hein, 2000). The Athabasca oil sands have been known for over
200 years and were originally named after the Athabasca River which passes through it and the evidence of heavy oils on the river banks (Carrigy, 1963).

Figure 1.6.1. Map of Canada highlighting the oil sands located in Alberta. Three major oil sands deposits are located in Alberta: Peace River, Cold Lake and the largest reservoir of crude bitumen in the world, the Athabasca deposit (Radler, 2002).

The Athabasca oil sands are 75%-80% inorganic materials (sand, clay and minerals) and 3-5% water with bitumen (semisolid mixture of complex hydrocarbons derived from coal or petroleum) content ranging from 10% to about 18% (Engelhardt and Todirescu, 2005; Budgell, 2006). The Athabasca oil sand deposits are found within beds of Lower Cretaceous period (Carrigy, 1963). The origin of the oil deposits has been debated by geologists for more than fifty years with most explanations involving marine source rocks (Stanton, 2004). However, prevailing theories suggest that the oil sand’s crude bitumen originated from non-marine, Carboniferous shales (359 - 300 million years ago) through the process of coalification (Stanton, 2004). Coalification is a temperature-pressure process where peat is changed to coal, and
eventually with increased temperature, there is a release of water and volatiles as coal progresses
to a bituminous rank (Stanton, 2004). Alberta’s estimated two trillion barrels of oil sand bitumen
is bordered by at least 650 billion tonnes of coal in the mountains and adjacent regions, so it is
proposed that the oil originated from Lower Cretaceous sediments through the process of
incomplete coalification (Stanton, 2004).

Two key characteristics of these oil sands make them economically recoverable, the area is
relatively shallow and the bitumen is encapsulated by water molecules (Engelhardt and
Todirescu, 2005). Escalating prices of oil and government assistance also help to make
extraction economically feasible. Alberta’s oil sands are one of the few oil deposits in the world
with growing production and about 176 billion barrels of proven oil reserves and a total
recoverable oil reserve estimated to equal almost 335 billion barrels (Engelhardt and Todirescu,
2005).

1.6.2. Oil extraction process

The mining of oil sands involves the removal of the bitumen-rich sand using open pit mining
methods, which is the most efficient method of extraction when there are large shallow deposits.
Another method involves processing the oil sand deposit in situ, where the bitumen is removed
while the sand remains in place (used for oil sands that are too deep to support surface mining
operations) (Budgell, 2006). For most in situ technologies the supply costs are about $13-$20
CAN to produce a barrel of bitumen which is marginally cheaper than the open pit mining
methods (Engelhardt and Todirescu, 2005).

Oil sands are recovered from mined or in situ formation by similar procedures. These
involve an initial emulsification of the oil with aqueous alkali, usually sodium hydroxide
solution, at a pH above about 11 (Chalaturnyk et al., 2002). The sand and bitumen is separated by adding warm water and agitating the mixture so that coarse material is removed. The mixture is then fed into a separation tank where the sand settles to the bottom and the mixture of sand, water and bitumen (called middlings) remains suspended in the middle layer. The impure bitumen froth floats to the top layer and is removed for further processing while the sand at the bottom of the tank is pumped into tailings ponds and the middlings go through a secondary separation process. The froth is diluted with naphtha (a volatile, liquid hydrocarbon solvent) to decrease its viscosity (Hein, 2006). Water and solids remaining in the bitumen froth are removed using centrifuges and settling units. The remaining bitumen is then converted to synthetic crude oil (Budgell, 2006).

Oil sand mining methods are damaging to the environment as they involve physical disturbances such as removal of trees and animals as well as the use of local water. In addition, hydrocarbon residuals and alkali are produced as a by-product after the extraction process. Oil sand production in Canada is the leading consumer of natural gas in Canadian industry and is also the largest contributor to greenhouse gas emissions. In 1967 the first commercially viable operation began and years of exploitation since in the Athabasca oil sands have resulted in the production of massive amounts (>50 square kilometers) of tailing sands. Oil companies that use strip mining methods to extract oil are required to remediate the area to its original environmental condition once the mining is completed. However, true remediation may pose an impossible goal as some land that was once forest may never be fully restored.
1.6.3. Current remediation process in Athabasca oil sands

Tailing sands already cover an area of land greater than 50 square kilometers in Alberta. As a by-product of the oil sands extraction process, the tailings, a mixture of water, sand, silt and fine clay particles, are pumped to a settling basin after bitumen extraction. Tailings sand settles out and is used to create dykes around ponds. The remaining fine tailings gradually solidify to form mature fine tailings (Chalaturnyk et al., 2002). Oil companies are expending considerable effort to overcome the challenges associated with tailings disposal and ultimate site reclamation because tailings sands pose a number of environmental risks including the migration of pollutants into the groundwater system and the leakage into the surrounding soil and surface water (Price and Adam, 2008).

Traditional remediation methods of tailing sands are costly and time consuming. The cost of remediation per hectare per year is around $2000 CAN and on a 10 year remediation plan can average about $20,000 CAN (which equals roughly $2 per barrel) (Lawrence, 2008). Compared to traditional methods (mixed fertilizer with tailing sands), bioremediation such as using microorganism, fungi and green plants to reclaim tailing sands appears to be more economic and safe. According to the reclaim standard, a reclaimed growing medium should be able to support a healthy plant community (similar to that of a comparable natural area) (Eschard and Huc, 2008). Developing new vegetation on tailing sands is the key to a successful remediation.
1.6.4. Soil environment of tailing sands

A reclaimed growing medium should be able to support a healthy plant community, but due to the processes involved in bitumen extraction, tailing sands contains polycyclic aromatic hydrocarbons (PAHs) and naphthenic acids (NAs) (Gentes et al., 2006) that may inhibit water absorption. In addition, mineral content is removed from the original oil sands by the extraction process (Eschard and Huc, 2008), the hot water and alkali involved in the extraction process may also kill what little soil microflora originally existed, and the high pH of tailing sands inhibits fungal growth (Kernaghan et al., 2002). Because of the reasons above, tailing sands are a challenging material to reclaim.

1.7. Study objectives

The main objective of this research was to observe the major fungi species associated with pioneer plants that could invade and colonize extracted oil sand sites. The second objective was to see if these fungi can confer stress tolerance in other plant species, thereby allowing the host plants inoculated with the specific fungal strains to grow in environments with similar abiotic stress factors.

1.7.1. What fungi are present in Taraxacum and Sonchus plant species?

Under the conditions with abiotic stress factors (low mineral content, hydrocarbon residual and high pH), pioneer plants such as Taraxacum and Sonchus still colonize on tailing sands. Because endophyte fungi have been shown to confer stress tolerance to host plants, endophyte fungal cultures were isolated from the samples of Taraxacum and Sonchus plants to examine the
type found in the samples of extracted, remediated, and unimpacted treatments. AMF abundance inside root of *Taraxacum* and *Sonchus* plants of extracted, remediated and unimpacted treatments were also detected and compared since they have been shown to assist plants and increase mineral nutrient uptake.

### 1.7.2. What type of fungi is more practical in assisting the remediation process?

The application of endophyte fungi in the bioremediation process of Athabasca oil sands is expected to be more convenient and economic than using AMF. Compared to AMF, which are obligate biotrophs, class 2 endophyte fungi can be easily recovered and cultured on artificial medium. These isolates can be applied to plants directly to study whether they confer stress tolerance to their host plants. Because the tailing sands are of low nutrient content and contain a hydrocarbon residual which makes the soil of extracted treatment hydrophobic, it forms a good high-stress environment model.

### 1.7.3. Can endophyte fungi confer tolerance to stress on tailing sands to tomato plants?

Research has shown that class 2 endophyte fungi confer environment stress tolerance to their host plants (Rodriguez and Redman, 2007) and are of vast host range. This knowledge was applied to test whether the same endophyte fungi isolated from tailing sands pioneer plants could confer stress tolerance in a model plant, the tomato (*Lycopersicon esculentum* Mill.).
1.7.4. Endophyte species characterization

Identifying species of endophyte fungi isolated from pioneer plants which confer stress
tolerance to host plants is important to see if they have been previously described in other host
plants and have a vast host range. Understanding this becomes important for the potential
application of the endophyte fungi in other high-stress environments besides the extracted tailing
sands. Species identification also helps us to further understand the interaction between
endophyte fungi and their host plants.

II. MATERIALS AND METHODS

2.1. Sample design

Five dandelion (*Taraxacum officinale*) samples and two soil samples were collected by
Blair Hersikorn and Judit Smits (Department of Veterinary Pathology, Western College of
Veterinary Medicine, University of Saskatchewan) in July, 2007 from each of the three kinds of
treatments (extracted, unimpacted and remediated) in the Athabasca oil sands. Although the
geographic locations of the samples were not clear and the number of collecting sites was
unknown, the samples were treated as replicates. Since whether they are really independent
replicates or not were not sure, the statistics must be treated as preliminary. Samples of the
extracted treatment were collected from sites that were covered by tailing sands (a by-product of
oil extraction). Samples of the unimpacted treatment were collected from sites that had not been
disturbed by oil extraction. Samples of the remediated treatment were collected from sites that
had already been reclaimed by adding fertilizer to tailing sands artificially after oil extraction.
2.2. Soil analysis

The following preparations (except the phosphorus preparation method) were taken and modified from the Department of Soil Science (University of Saskatchewan) general protocol (de Freitas, 2007). All soils were air-dried and ground before analysis and two soil samples from each treatment were measured.

2.2.1. Measurement of soil pH

Soil pH was measured in 18 Megohm double deionized water (ddH2O) as it most accurately reflects the pH of soil solution in the field, especially for unfertilized soils (Hendershot et al., 1993). A 20 g sample of air-dried soil was put into a 100 mL glass beaker. Forty mL of 18 Megohm ddH2O was added and shaken on a rotary shaker at 142 rpm for 20 min. The suspension sat for 1 h, then was filtered through Whatman #1 filter papers into 28 mL vials. All pH readings were measured using a pH meter (VWR 8100 model), calibrated with standard solutions of pH 4.0 and pH 7.0. Three measurements of pH were taken for each of soil sample. Results were
reported as the mean ± standard error of the three measurements of each sample for each treatment.

2.2.2. Measurement of soil organic carbon

Ten grams of soil samples were air dried at room temperature for 3 d. Dry soil was ground into fine powder using a mortar and pestle, then passed through a 12-mesh (approximately 2 mm) screen. Samples were weighed and approximately 0.2 g of ground soil samples were placed into a Leco CR-12 Carbon determinator (LECO Corporation, St. Joseph, MI, USA http://www.leco.com/index4.htm). Detection range of the Leco CR-12 Carbon determinator was 0.005-50%. Organic carbon was measured by combustion at 840 °C in a pure oxygen atmosphere. Soil organic C content of less than 0.2 g of soil samples can be completely oxidized within 2 min. The organic matter was converted to water and CO₂. Water was absorbed by magnesium perchlorate and the CO₂ was detected by an infrared detector. Organic carbon content was automatically calculated based on the amount of CO₂ detected by the infrared detector. Soil organic C content was reported as a percentage (%) in the soil. There were three sub-samples for each sample. For quality assurance and quality control of this test, sucrose (containing 42.1% organic carbon) was used as a certified reference material (used after every 10 tests). Results were reported as the mean ± standard error of the three measurements of each sample for each treatment.
2.2.3. Measurement of soil K, Ca, Mg, Na

Ten grams of soil samples were air dried at room temperature for 3 d. Dry soil was ground into fine powder using a mortar and pestle then passed through a 12-mesh (approximately 2 mm) screen. One gram of dried ground soil samples were placed into 50 mL plastic centrifuge tubes. Forty milliliters of 1 M ammonium acetate solution was added and the mixture was shaken on a rotary shaker at 142 rpm for 1 h. The suspension was filtered through VWR 413 filter paper into 28 mL vials and the soil solution was analyzed in an atomic absorption spectrophotometer (Spectr AA 220, VARIAN, Inc., www.varianinc.com) which is a common technique used for determining the concentration of a particular element in a given sample (Welz, 1999). Detection ranges of different elements are as following: K-0.03-2 ppm, Ca-0.01-3 ppm, Mg-0.003-1 ppm, Na-0.002-1 ppm. Wavelength of light used for measurement of K, Ca, Mg, Na content are 766.5 nm, 422.7 nm, 285.2 nm and 589 nm respectively.

The general principle of this method rests on the fact that electrons of atoms can be promoted to higher orbitals by absorbing light of a given wavelength. Because each wavelength is specific to a particular electron transition in a particular element, it is possible to determine the amount of an element in a given sample. This technique involves the use of interchangeable filters to select light with element specific wavelengths, a flame or a furnace apparatus for volatilizing the sample, and a photon detector. Different filters are chosen that select a light that is absorbed by a specific element and the amount of light absorbed depends of the amount of element in the sample. Results were reported as mg/L in the soil. Quality control and assurance were maintained by using certified standards and appropriate method blanks.
2.2.4. Measurement of soil available N

Nitrogen, pooled together in the forms of ammonium, nitrate, and nitrite ions, was extracted from the soil samples and measured as total available N content. Five grams of dried ground soil was weighed out into 100 mL glass beakers and 50 mL 2 M KCl solution was added. The mixture was shaken on rotary shaker at 142 rpm for 1 h and the suspension was filtered through VWR 413 filter paper into 28 mL vials. The N content in the soil samples (two samples of each treatment) was measured in an automatic analyzer by the ALS Laboratory Group, Saskatoon, SK. Results were reported as mg/L in the soil.

2.2.5. Measurement of Soil Extractable P

Soil P was extracted as described by McGonigle and Miller (1996). Extracts were prepared from 2 g of soil shaken for 30 min with 40 mL 0.5 M NaHCO_3, pH 8.5 and filtered through Whatman No. 2 paper to remove soil. The filtered extracts were sent to the ALS Laboratory Group, Saskatoon, SK. Extractable P content was measured using inductively coupled plasma atomic emission spectroscopy (ICP-AES) at 882 nm there. ICP-AES is an emission spectrophotometric technique, exploiting the fact that excited electrons emit energy at a given wavelength as they return to ground state. The fundamental characteristic of this process is that each element emits energy at specific wavelengths peculiar to its chemical character. Although each element emits energy at multiple wavelengths, in the ICP-AES technique it is most common to select a single wavelength (or a very few) for a given element. The intensity of the energy emitted at the chosen wavelength is proportional to the amount (concentration) of that element in the analyzed sample. Thus, by determining which wavelengths are emitted by a
sample and by determining their intensities, the analyst can quantify the elemental composition of the given sample relative to a reference standard. Results were reported as mg/L in the soil.

2.2.6. Soil hydrophobicity

The hydrophobicity assay was assessed by placing 20 μL drops of distilled water onto screened soil samples prepared as in section 2.2. Pictures were taken to record the level of hydrophobicity for different soil samples. Soils were judged to be hydrophobic if the water drop remained on the surface of the soils instead of being absorbed by the soils immediately after application.

2.3. Detection of AMF and endophytic fungi in plant tissue by wide field epifluorescence microscope and CLSM

Five dandelion (Taraxacum officinale) samples from each of the three treatments (extracted, unimpacted and remediated) were examined. Randomly selected lateral roots (Figure 2.3) of each dandelion samples were cut into 1 cm segments and cleared by autoclaving for 20 min in 10 % KOH. The KOH was removed with two washes in room temperature by 70 % ethanol. Cleared samples were stained for 3 h at 68 °C in 0.05 % acid fuchsin in 85 % lactic acid (Allen et al., 2006; Kaminskyj, 2008). Excess stain was removed in two changes of 1:1:1 distilled water: 85% lactic acid: glycerol. Then samples were kept in the distaining solution in a 47 °C water bath overnight. The stained root samples were mounted in polyvinyl alcohol (PVAG) medium (recipe below) and the PVAG medium was polymerized overnight at 40 °C. Edges of the cover slip were sealed with transparent nail polish. Samples were observed using
a wide field epifluorescence microscope, the Zeiss META 510 confocal laser scanning microscope (Allen et al., 2006; Kaminskyj, 2008).

Figure 2.3. Lateral roots of dandelion (Taraxacum officinale). The dandelion shown in this figure was collected at the University of Saskatchewan.

The PVAG medium used in this step was modified from Brundrett et al (1996). PVAG medium contains 4 g polyvinyl alcohol powder (http://www.vwrcanlab.com/ JT Baker, VWR International, Edmonton, AB), 50 mL distilled water and 20 mL glycerol. This mixture was warmed to 60 °C with constant stirring and covered with a cap until it was totally dissolved (4 h to overnight). The solution will solidify and opalescent in room temperature, but it can still be re-melted with gentle heat and stirring (Kaminskyj, 2008).

Lactofushsin (LF) used for staining in this step is commonly used in the staining of plant samples to study the morphology of AMF and fungal endophytes as LF can bind to chitin, a component of most fungal walls (Bevege, 1968; Kormanik et al., 1980). LF has a wide range of
excitation wavelengths, spanning at least 405-534 nm (blue to green) available with most epifluorescence systems (Kaminskyj, 2008).

The wide field epifluorescence microscope used for endorhizal quantitation was a Zeiss Axioplan microscope equipped a 20× N. A. 0.5 Plan Neofluar, a 40x N.A. 0.75 Plan Neofluar, and a 63x N. A. 1.4 Plan Apochromat oil immersion objective. Images of lactofuchsin-stained material were collected through a BP546 excitation filter, FT580 dichroic mirror, and LP590 emission filter. Epifluorescence microscopy is a method where excitatory light is passed from above, through the objective and then onto the specimen instead of passing it through the specimen. Because the LF stain is a very stable fluorochrome, photobleaching is seldom a problem (Kaminskyj, 2008). Wide field epifluorescence microscopy lights up the entire field, provides an improved signal to noise ratio and is an efficient and flexible method for quantification (Kaminskyj, 2008).

A Zeiss META 510 Confocal laser scanning microscope (CLSM) (www.zeiss.com) equipped with 25× Plan NeoFluar N. A. 0.8 and 63× C-Apochromat N. A. 1.2 multi-immersion objectives was used for imaging the stained samples. Images were collected at the setting of 543 nm excitation, 9.9 % intensity of a 25 mW beam from a HeNe laser, a HFT 488/543 beam splitter, and a 604-657 nm emission filter. Fluorescence and transmitted light images were collected at the same time. CLSM provides better images of 2D and 3D objects than can be obtained with conventional optical microscopes. CLSM collects optical sections from thick specimens and uses spatial filtering to eliminate out-of-focus light or flare in specimens that are thicker than the plane of focus. (Allen et al., 2006; Ormsby et al., 2007; Kaminskyj, 2008)
2.4. Analysis of fungal abundance

Fungal colonization of root systems was assessed using the Multiple Quantitation Method (MQM) (Ormsby et al., 2007) and LF stained roots. Roots were examined by using wide field epifluorescence microscopy. Intersections were examined one field of view (1 mm) apart at 200x and 400x magnifications (Figure 2.4.1). Each intersection was assessed for the following types of endorhizal fungi: 4-6 μm wide aseptate hyphae characteristic of AM; arbuscules and vesicles associated with AM hyphae; 1-1.5 μm wide aseptate hyphae characteristic of FE; arbuscules and vesicles associated with FE; SE hyphae; and hyphal coils. Intersections not associated with any fungi were also recorded to provide an estimation of total colonization. One hypha, 2-5 hyphae and 6 hyphae per intersection were recorded as low, medium and high levels of colonization respectively. Five plant samples of each treatment were assessed, that is 52-56 intersections of each plant sample were tested. Results were expressed as mean ± standard error of the mean of the five samples and statistically analyzed by one-way ANOVA in SPSS. Significance was assessed at P < 0.05.

Figure 2.4.1. The scanning procedure for the Multiple Quantitation Method. Roots (A) are visualized using at 200x and 400x magnifications (B). Fungal structures that intersect the vertical line on the graticule are considered, throughout the entire focal depth of the root. (C) Intersections are evenly spaced by moving the stage by one field of view each time (Kaminskyj, 2008).
2.5. Recovering endophytic fungi from *Taraxacum* and *Sonchus* tissue

In July 2007, two dandelion (*Taraxacum officinale*) samples of each treatment (extracted, unimpacted and remediated) and four sowthistle (*Sonchus arversis*) samples of extracted treatment were collected by Dr. Ali Quoreshi (Symbiotech, Niski, AB) in the Athabasca oil sands. Plants were washed until the soil was removed, then surface sterilized in 1 % (v/v) sodium hypochlorite solution for 3 min (Rodriguez *et al.*, 2008b). Using aseptic technique, plants were cut into sections of 2-3 cm lengths from the roots, leaves and stems separately, plated on 10 % PDA in Petri dish and incubated for 5-7 days at 28 °C to allow for the emergence of fungi.

2.6. Purification and morphological identification of endophytic fungi strains

Endophytic fungi were recovered from plant materials (roots, leaves and primarily stems) collected from each soil type. After the primary fungi cultures had sporulated, endophytic fungi isolates were subcultured on a 10 % PDA medium (contains 3.9 g PDA powder and 12 g agar per liter) according to colony color, texture, habit, and growth rate. In order to develop a pure culture of the endophytic fungi strains from the environmental samples, a sterilized loop was used to collect a tiny amount of spores. The spores were then spread onto fresh 10 % PDA plates, which were incubated at 28 °C for 2-3 d to allow isolated single-spore colonies to emerge. The single spore colonies were then selected and subcultured. For morphological identification, a piece of agar carrying fungal tissue was cut from the pure endophytic fungi culture and a clean coverslip was placed on top of the side with fungal tissue, then incubated at room temperature in a Petri dish with distilled water. The fungi on the agar piece were allowed to grow on the coverslip until they begun to sporulate. Coverslips were mounted in lactophenol and sealed with transparent nail polish. These slides were labeled and later observed under a compound
microscope for traditional morphological identification according to what has been previously described by Ellis and Ellis (1985). Pictures of fungi were captured by Sensys CCD (www.roper.com) driven by ImageManager RSI under compound microscope.

2.7. Plant colonization

Tomato (Lycopersicon esculentum Mill.) plants were chosen to inoculate with isolated endophytic fungi because tomato plants are a model plant system of known genome sequence and are easy to grow in a greenhouse environment. Tomato plants also have a relatively high requirement for nutrients and water, making it sensitive to environmental stress factors such as drought and low nutrition. Tomato seeds (Lycopersicon esculentum Mill.) obtained from plants grown in Sunshine #1 potting mixture (Sun Gro Horticulture, USA), hereafter called potting mixture, under 8 h light/16 h dark within a growth chamber at a temperature regime of 25 °C Light/ 23 °C dark (Rastogi and Sawhney, 1990) were supplied by Dr. Sawhney’s lab (Department of Biology, University of Saskatchewan). Seeds were surface sterilized in 50 mL of 1% (v/v) sodium hypochlorite for 20 min with moderate agitation and rinsed with 10-20 volumes of sterile 18 MΩ water. Seeds were germinated on 10 % PDA medium and maintained in incubator for 5-7 d. To ensure this study began only with axenic plants, seedlings (ranging in size from 0.5-4 cm from cotyledons to tip of radicle) that showed no outgrowth of fungi into the surrounding medium were chosen and transplanted; any seedlings showing an outgrowth of fungi were discarded. Spores of putative endophytic fungi were harvested in 18 MΩ water by rubbing the surface of a sporulating pure culture with a sterile bent glass rod. Spore density was estimated using a hemocytometer and compound microscope (200x total magnification). The spore suspension was diluted in sterile 18 MΩ water to prepare 10^4-10^5 spore mL^{-1}. The axenic
tomato seedlings were inoculated with endophytic fungi by making the seedlings immersed in 15-20 mL of spore solutions (10^4-10^5 mL^-1) in Petri dish for 30 min. Double-decker magenta boxes (65 mm × 65 mm × 100 mm) supplied by Dr. Bonham-Smith (Department of Biology, University of Saskatchewan) were filled with equivalent amounts (50 ± 0.5 g) of potting mixture in the upper chamber. The lower chamber was filled with 250 mL of plant food solution (Plant-Prod® All Purpose 20-20-20 Water Soluble Fertilizer, Brampton, Ontario, www.plantprod.com). As the plant food solution was prepared as 1 g/L, it contained 200 ppm Nitrogen (N), 200 ppm Phosphoric Acid (P2O5) and 200 ppm soluble Potash (K2O), in addition to 0.2 ppm Boron (B), 0.5 ppm Cu, 1 ppm Fe, 0.5 ppm Mn, 0.005 ppm Mo, 0.5 ppm Zn, and 1 ppm EDTA (http://www.plantprod.com). The solution was added with 5 mM CaCl₂ to stimulate endophytic fungi to colonize the plants (Rodriguez and Redman, 2007). Then the double-decker magenta boxes were sterilized by autoclaving for 22 min at 121 °C. Axenic and symbiont-inoculated seedlings were planted in the double-decker magenta boxes after they cooled down to room temperature.

2.8. Abiotic stresses assay

Low-nutrition: After two weeks growth on potting mixture, axenic and symbiont-inoculated plants were transplanted to sterile double-decker magenta boxes containing equivalent amounts (150 ± 5 g) of sterilized tailing sand. The lower chamber was filled with 250 mL of sterilized distilled water. After another two weeks growth of the plants in the greenhouse, the relative plant health of both of axenic plants and symbiont-inoculated plants was recorded by photograph and quantified according to wet and dry weight. Ten independent replicates of plant samples were taken for each treatment type.
Drought: After axenic and symbiotic plants grew on potting mixture for 2 weeks, the fluid in the lower chamber of the double-decker magenta box was poured out and the plant soils dried out over time. Soil samples were collected from the upper chamber of the double-decker magenta boxes once the tomato plants started to wilt. The soil samples were ground and weighed (W1) then dried out at 39 °C for 72 h. Dried soil samples were weighed again (W2). Moisture content of each soil sample was calculated according to the following formula: Water percentage \( = \frac{(W1-W2)}{W1} \times 100 \% \) (Table 3.5.2). Five independent replicates of soil samples were taken for each treatment type.

2.9. Reisolation and identification of endophytic fungi from inoculated plant tissue

Plants that inoculated with isolated endophytic fungi strains were harvested and washed until potting mix or tailing sands residue was removed, then surface sterilized in 1% (v/v) sodium hypochlorite solution for 3-5 min (Rodriguez et al., 2008b). Using aseptic technique, plants were cut into sections representing the roots, leaves and stems, plated on 10% PDA in Petri dish and incubated for 5-7 d at 28 °C to allow for the emergence of fungi.

2.10. DNA extraction

Approximately 100 μL of sand was added to a 1.5 mL microfuge tube with 500 μL of lysis buffer (contains 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA). A pea-sized amount of mycelium was taken from the plate with a sterile toothpick and added to the buffer/sand mixture. The mycelium was ground with a small pestle and this mixture was vortexed for 3 min on high speed. The tube was centrifuged on high for 1 min. Then the
supernatant was transferred to a new tube. After 500 μL chloroform was added to the tube, the solution was vortexed for 1 min and then centrifuged on high for 1 min. The top layer (~300 μL) was then carefully transferred to a new tube. One tenth volume of 3 M sodium acetate and 1 volume isopropanol was added to the sample and mixed well by inversion. This was left at room temperature for 15 min and then centrifuged at 13000 rpm for 15 min. The supernatant was discarded and the DNA pellet was rinsed with 500 μL 70 % ethanol. Finally, the DNA pellet was air dried for 10 min, then dissolved in 50 μL ddH₂O.

2.11. Polymerase chain reaction and sequence

Species designations were based on sequences analysis of the variable ITS1 and ITS2 sequence of rDNA. Primer pair ITS4 and ITS5 (sequences shown in Table 2.11) were used for the amplification of target sequence. PCR reactions were performed in a final volume of 50 μL containing PCR buffer (10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris-HCl, pH 8.8, 0.1% Triton X-100), 1.5 mM MgCl₂, 200 μM each of dATP, dCTP, dGTP, dTTP, 15 pmols of each primer, 2.5 units of Taq DNA polymerase and 100 ng of fungal genomic DNA used as template DNA.

Amplification was performed in a TECHNE Genius thermal cycler (www.techneusa.com) programmed as follows: Initial denaturation cycle at 94 °C (3 min), followed by 35 cycles of denaturation at 94 °C (20 s), annealing at 52 °C (50 s) and extension at 72 °C (1 min). The last cycle was followed by 10 min at 72 °C. For each PCR reaction, 4 μL of PCR products were separated by electrophoresis on a 1.0 % agarose gel (containing 0.5 mg L⁻¹ ethidium bromide) for 30 min at 90 Voltage in 1×Tris-acetate buffer with 40 mM Tris-acetate and 1 mM EDTA, pH 8.3 (Sambrook et al., 1989) The PCR products were revealed under UV light. The target PCR product bands were cut from the agarose gel under UV light and purified by using QIAquick Gel
Extraction Kit (http://www1.qiagen.com/, Qiagen Inc., Stanford, Valencia, CA, USA). The concentration of the purified DNA products was measured by using UV spectrophotometer according to the absorbance at 260 nm as follows: 1) One μL DNA sample was diluted in 99 μL ddH₂O; 2) the UV disposable cuvettes were filled with 100 μL distilled water as blank to adjust the OD reading of the UV spectrophotometer to be 0; 3) the distilled water was discarded and 100 μL of diluted DNA sample was put into the dried clean cuvette; 4) the OD value of DNA sample was read at 260 nm. Because 50 μg / mL of DNA concentration (in water) has an absorbance of 1.0 at 260 nm, the concentration of the tested DNA sample can be calculated according to the formula that concentration of DNA = OD × 50 μg / mL × 100. The purified DNA fragments were sequenced at Plant Biotechnology Institution (PBI), sequencing facility, Saskatoon, SK. The sequence data were BLAST searched against the GenBank database (www.ncbi.nlm.nih.gov/BLAST/index.shtml).

To identify the Trichoderma spp. at species level, in Dr. Samuels’ lab, PCR amplification of the internal transcribed spacer region (ITS1, 5.8 S and ITS2), a 0.65 kb section of translation elongation factor (EF-1α), 0.5 kb section calmodulin gene (cal), and 0.75 kb actin gene (act) was performed in a 50 mL reaction volume containing 5 mL of 10 x PCR buffer (New England Biolabs, Ipswitch, MA), 200 mM dNTPSs (Promega, Fitchburg Center, Wisconsin, USA), 0.2 mM of each primer, 1.25 units of Taq Polymerase (New England Biolabs) and 10–50 ng of template DNA. The primers used for amplification of ITS were ITS1 and ITS4 (White et al. 1990). For EF-1α the primers were EF1-728F (Carbone and Kohn, 1999) and TEF1 rev (Samuels et al., 2002). For cal the primers were CAL-228F and CAL737R (Carbone & Kohn 1999). For act the primers were Tact1 and Tact2 (Samuels et al., 2006). The sequences of all of the primers above are shown in Table 2.11. The program used for amplification of all the genes consisted of
3 min initial denaturation at 94°C followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 50°C for 30 s and extension at 72 °C for 1 min and then a final 10 min extension. The PCR products were purified using Qiagen QIAquick PCR purification kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions then sent to sequence.

### Table 2.11. Primers used in PCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’-3’)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS4</td>
<td>5’-TCCTCCGCTTTATTGATATGC-3’</td>
<td>58</td>
</tr>
<tr>
<td>ITS5</td>
<td>5’-GGAAGTAAAGTGCTGTAACAAGG-3’</td>
<td>53</td>
</tr>
<tr>
<td>ITS1</td>
<td>5’-TCCGTAGGTGAAACCTGCGG-3’</td>
<td>57</td>
</tr>
<tr>
<td>EF1-728F</td>
<td>5’-CATCGAGAAGTTGAGAAG-3’</td>
<td>58</td>
</tr>
<tr>
<td>TEF1 rev</td>
<td>5’-GCCATCCTTGGAGATACCAGC-3’</td>
<td>61</td>
</tr>
<tr>
<td>CAL-228F</td>
<td>5’-GAGTTCAAGGAGGCCCTTCTCCC-3’</td>
<td>63</td>
</tr>
<tr>
<td>CAL737R</td>
<td>5’-CATCTTTCTGGCCATCATGG-3’</td>
<td>60</td>
</tr>
<tr>
<td>Tact1</td>
<td>5’-TGGCACCACACACCTTTCATAATGA-3’</td>
<td>63</td>
</tr>
<tr>
<td>Tact2</td>
<td>5’-TCTCCTTCTGCATAAGGTCGGA-3’</td>
<td>64</td>
</tr>
</tbody>
</table>

### III. RESULTS

#### 3.1. Soil assay

The results suggest soil samples of extracted treatment were generally of the lowest nutrition content (except N and perhaps K), lowest organic C content, and highest pH compared to soil samples of remediated and unimpacted treatment in this assay (Table 3.1.1). The hydrophobicity test showed that water soaked into unimpacted soil as soon as it was applied, but
water beads remained on the surface of soil samples of extracted and remediated treatments long enough for a picture to be taken (at least 5 seconds) (Figure 3.1.1). This result suggests that the soil samples of extracted and remediated treatments were relatively hydrophobic compared to the unimpacted soil. However, due to the limitation of sample size and the unclear collecting sites, these results should be treated with caution.

Table 3.1.1. Soil test data obtained from unimpacted, extracted, and remediated treatments

(2 soil samples of each treatment)

<table>
<thead>
<tr>
<th></th>
<th>Available N (ppm)</th>
<th>Available P (ppm)</th>
<th>Calcium (ppm)</th>
<th>Magnesium (ppm)</th>
<th>Sodium (ppm)</th>
<th>Potassium (ppm)</th>
<th>Organic Carbon (%)</th>
<th>pH</th>
<th>Hydrophobicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unimpacted</td>
<td>0.5</td>
<td>0.7</td>
<td>0.9</td>
<td>4.7</td>
<td>86</td>
<td>155.1</td>
<td>9.0 ± 0.2*</td>
<td>1.9 ± 0.2*</td>
<td>No</td>
</tr>
<tr>
<td>Extracted</td>
<td>0.7</td>
<td>0.5</td>
<td>0.1</td>
<td>0.1</td>
<td>4.5</td>
<td>4.1</td>
<td>0.2 ± 0.0*</td>
<td>0.2 ± 0.0*</td>
<td>Yes</td>
</tr>
<tr>
<td>Remediated</td>
<td>1</td>
<td>0.7</td>
<td>0.7</td>
<td>0.1</td>
<td>145.9</td>
<td>165</td>
<td>4.2 ± 0.1*</td>
<td>3.0 ± 0.1*</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* Values in Table 3.1.1 represent mean and standard error of 3 measurements per sample.

Hydrophobicity differences were obtained qualitatively (Figure 3.1.1).
Figure 3.1.1. Hydrophobicity assay showing (from top to bottom) soil samples of unimpacted, extracted and remediated treatments. Soil samples of remediated treatment (bottom right) and extracted treatment (middle right) are relatively hydrophobic compared to soil samples of unimpacted treatment (top right).

3.2. Endorhizal fungi structures observed and relative abundance in dandelion (*Taraxacum officinale*) samples from Athabasca oil sands

Many different endorhizal fungi were found in the dandelion (*Taraxacum officinale*) samples growing upon tailing sands. AMF are characterized as having 4-6 μm wide aseptate hyphae and large spores (Figure 3.2.1 A) as well as fine-branched arbuscules (Figure 3.2.1 B). AMF hyphal coils were also observed in root samples from tailing sands as Figure 3.2.1 C
shows. Fine endophytes (FE) (Figure 3.2.1 D) are characterized as the diameter of hyphae less than 2 μm. Septate endophytes (SE) (Figure 3.2.2 A) were distinguished by the septa structures in hyphae. Dark septate endophyte (DSE) (Figure 3.2.2 B), which cannot be stained by lactofuchsin, were also found in *Taraxacum officinale* root samples.

A one-way ANOVA showed there was no significant difference between the colonization percentage of AMF, SE, FE, and total fungi in *Taraxacum officinale* samples of unimpacted, extracted, and remediated treatments (Table 3.2.1).
Figure 3.2.1. AMF and FE endorizal fungi in lactofuchsin-stained dandelion lateral roots from tailing sands viewed by CLSM. A-D represent AMF and FE fungi types, a natural group often associated with each other. A) AMF hyphae of 4-6 μm diameter and AMF vesicle found in dandelion lateral roots grown in tailing sands; B) Abundant arbuscules found in dandelion lateral roots grown in tailing sands; C) AMF hyphal coil observed in dandelion lateral roots grown in tailing sands; D) FE hyphae of less than 2 μm diameter found in dandelion lateral roots grown in tailing sands.
Figure 3.2.2. SE and DSE fungi in LF-stained dandelion lateral roots from tailing sands viewed by CLSM. SE and DSE fungi types form another natural fungi group. A) Septa of SE fungi found in dandelion lateral roots grown in tailing sands; B) Hyphae of DSE found in dandelion lateral roots grown in tailing sands.
Table 3.2.1. Endorhizal fungal colonization (% abundance) of dandelion roots subjected to different treatments (n=5, values represent mean of 5 samples ± standard error)

<table>
<thead>
<tr>
<th></th>
<th>Arbuscular mycorrhizal fungi</th>
<th>Septate endophyte fungi</th>
<th>Fine endophyte fungi</th>
<th>Total fungal colonization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unimpacted</td>
<td>70.8±9.3</td>
<td>38.3±7.0</td>
<td>19.0±7.5</td>
<td>84.0±4.8</td>
</tr>
<tr>
<td>Extracted</td>
<td>76.4±8.5</td>
<td>21.3±11.4</td>
<td>15.8±13.9</td>
<td>89.4±5.6</td>
</tr>
<tr>
<td>Remediated</td>
<td>83.4±4.3</td>
<td>23.6±10.0</td>
<td>19.2±6.5</td>
<td>95.5±1.1</td>
</tr>
<tr>
<td>P-Value</td>
<td>0.744</td>
<td>0.502</td>
<td>0.950</td>
<td>0.207</td>
</tr>
</tbody>
</table>

Percent abundance assay of endorhizal fungi in dandelion samples of all treatments. Total fungal colonization for each sample (5 slides for each sample) was calculated as: # of intersections containing fungal structures / Total intersections x 100%. Root samples were examined microscopically (400x) using the MQM method (Ormsby et al., 2007).

3.3. Recovery and morphological identification of endophytic fungi from *Taraxacum* and *Sonchus* samples

Different endophytic fungi strains were represented in samples of different treatments. Identification and isolation was based on characteristics of whole colonies (Figure 3.3.1) and spore morphology (Figure 3.3.2). *Trichoderma* represented in *Taraxacum* samples of extracted treatments (Figure 3.3.1) and a *Fusarium* isolate and a *Pseudeurotium* isolate were isolated from plants growing on remediated and unimpacted soil respectively (Figure 3.3.2). *Alternaria* strains were common from all types of treatments.
Figure 3.3.1. Fungal endophyte represented in *Taraxacum* and *Sonchus* samples from extracted treatments. A. *Trichoderma* spp isolated from *Taraxacum*; B. *Alternaria* spp isolated from *Sonchus*. 
Figure 3.3.2. Endophytic fungi isolated from *Taraxacum* and *Sonchus* showing characteristic spore morphologies. A. *Alternaria* spp. represented in samples from extracted treatments; B. *Trichoderma* spp. represented in samples from extracted treatments; C. *Pseudeurotium* spp. represented in samples from unimpacted treatments; D. *Fusarium* spp. represented in samples from remediated treatments.

3.4. Endophytic fungi colonization and abiotic stress treatment

Tomato plants inoculated with *Trichoderma* spores and axenic tomato plants were transplanted to tailing sands after 2 weeks growth on potting mixture (see Figure 3.4.1). After 2 weeks growth on tailing sands, tomato plants inoculated with *Trichoderma* spores grew better than axenic tomato plants. Table 3.4.1 provides quantification of this observation based on a comparison of fresh plant weight and dry plant weight. The drought test performed showed no
significant difference ($P = 0.155$) in average moisture content at the time of wilting in soil that supported the axenic plants and the soil that supported the *Trichoderma* inoculated plants (see Table 3.4.2). Although the average moisture content was higher in soil that supported the axenic plants, the statistical result suggests that this difference is no greater than would be expected by chance alone.

![Figure 3.4.1.](image)

**Figure 3.4.1.** Photographs of tomato seedlings in potting mixture after grown under 8 h light / 16 h dark within a growth chamber at a temperature regime of 25 °C Light / 23 °C dark (Rastogi and Sawhney, 1990) for 2 weeks. A. Axenic tomato plants; B. Tomato plants inoculated with *Trichoderma* spp. spores.
Figure 3.4.2. Photographs of axenic tomato plants (A) and tomato plants inoculated with *Trichoderma* spp. (B) after growing on tailing sands for 2 weeks. Tomato plants inoculated with *Trichoderma* spp. were growing much better and appeared to be more healthy (based on overall size and color) compared to the non-symbiotic tomato plants under the same conditions (soil, temperature, moisture and light).

Table 3.4.1. Weight (total biomass) of axenic plants and plants inoculated with *Trichoderma* after two weeks growth on tailing sands (*n*=10, values represent mean of 10 samples ± standard error)

<table>
<thead>
<tr>
<th></th>
<th>Fresh weight (g)</th>
<th>Dry weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axenic tomato plants</td>
<td>0.196±0.063</td>
<td>0.014±0.002</td>
</tr>
<tr>
<td>Inoculated tomato plants</td>
<td>0.560±0.047</td>
<td>0.026±0.003</td>
</tr>
<tr>
<td><strong>P-Value</strong></td>
<td><strong>0</strong></td>
<td><strong>0.001</strong></td>
</tr>
</tbody>
</table>

Test of fresh and dry weight (total biomass) of axenic tomato plants and tomato plants inoculated with *Trichoderma* spp. after two weeks growth on tailing sands showed tomato plants inoculated with *Trichoderma* spp. were of higher average fresh and dry weight compared to axenic tomato plants. Total biomass included roots, stems and leaves of each tomato plant. Two sample *t*-test showed a significant difference between axenic tomato plants and tomato plants inoculated with *Trichoderma* spp. in fresh weight and dry weight (*P* < 0.05).
Table 3.4.2. Comparison of soil moisture content at start of plant wilting in drought tolerance experiment
(n=5, values represent mean of 5 samples ± standard error)

<table>
<thead>
<tr>
<th></th>
<th>Soil moisture content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axenic tomato plants</td>
<td>22.92±2.42</td>
</tr>
<tr>
<td>Inoculated tomato plants</td>
<td>17.94±1.98</td>
</tr>
<tr>
<td>P-value</td>
<td>0.155</td>
</tr>
</tbody>
</table>

Drought test of axenic tomato plants and tomato plants inoculated with *Trichoderma* spp. Two sample t-test showed there is no significant difference between axenic tomato plants and tomato plants inoculated with *Trichoderma* spp. in the moisture content when the plants just started to wilt (P > 0.05). Soil moisture content was measured by weight.

3.5. Detecting endophytic fungi inside of plant tissue

Using CLMS and epiflorescent microscopy, it turned out to be impossible to detect endophytic fungi associated with the inoculated tomato plant even though data presented in Section 3.6 showed they could be reisolated using microbial technique. To show what endophytic fungi look like associated with their host plants, rice seedlings were chosen based on previous evidence that showed that rice seedlings can be inoculated with endophytic fungi that confer a stress tolerance (Rodriguez *et al.*, 2008b). As a monocotyledon, rice has different characteristics (such as vascular tissue organization) compared to the dicotyledon tomato plant. These differences may be responsible for the difficulty in being able to detect endophytic fungi associated with tomato plants using CLMS and epiflorescent microscopy.

Pictures of endophytic fungi in rice seedling (Figure 3.5.1) vividly show what endophytic fungi look like associated with monocotyledon plant roots. Rice seedlings inoculated with endophytic fungi from Rodriguez & Redman’s lab (University of Washington, Seattle, USA) were inoculated with a *Fusarium* spp. isolated from a salt stress habitat (Figure 3.5.1 A) and a
Curvularia spp. isolated from a thermal stress habitat (Figure 3.5.1 B). These two isolates were selected for demonstration as they are believed to confer a stress tolerance, similar in function to the endophytes used to inoculate the tomato plants.

![Image](image.png)

**Figure 3.5.1.** Confocal microscope pictures of fungi associated with roots of rice seedlings inoculated with *Fusarium* (A) and *Curvularia* (B) respectively.

### 3.6. Re-isolating inoculated endophytic fungi from tomato plants

Endophytic fungi colonized plants should contain a recoverable fungal strain which is identical to that used in the original inoculation. As shown in Figure 3.6.1, there were no fungi present in the axenic plant tissue (A), but there were specific endophytic fungi strains present in the inoculated plants (*Fusarium* spp. (B), *Trichoderma* spp. (C), and *Alternaria* spp. (D)). These results confirm that the plants inoculated did contain the specific endophytic fungi strains used and that there were no fungi in the axenic plants.
Figure 3.6.1. Pictures of fungi that had grown from axenic plants (A), and plants inoculated with *Fusarium* spp (B), *Trichoderma* spp. (C), and *Alternaria* spp. (D). Fungi were recovered on 10 % PDA medium after 5-7 days at 28 °C.

3.7. PCR products

rRNA-ITS gene fragments amplified with primers ITS4 and ITS5 from *Alternaria*, *Trichoderma* and *Fusarium* strains separated on agarose gel are shown in Figure 3.7.1. The marker used was 1000 bp and the PCR products of the endophytic fungi were around 600 bp, running on a 1% agarose gel. A *Trichoderma* strain isolated from dandelions that grew in tailing sands, which confer host plant’s tolerance to tailing sands, was selected and sequenced. The DNA sequence was compared to known DNA sequence data in Genbank. The rRNA-ITS DNA sequence of *Trichoderma* strain isolated from the dandelion was a 99 % match to *Trichoderma harzianum* (*Hypocrea lixii*), *Trichoderma atroviride*, *Trichoderma citrinoviride* and *Trichoderma inhamatum* according to the BLAST searching result. The *Trichoderma* strain was
finally identified as *Trichoderma harzianum* according to the sequence information of internal transcribed spacer region (ITS1, 5.8 S and ITS2), a 0.65 kb section of translation elongation factor (EF-1α), 0.5 kb section calmodulin gene (cal), and 0.75 kb actin gene (act) in Dr. Samuels’ lab.

**Figure 3.7.1. PCR results.** 1-1kb marker, 2&4 PCR products of *Alternaria* strains, 3&5 PCR products of *Trichoderma* strains, and 6 PCR products of *Fusarium* strains.

**IV. DISCUSSION**

4.1. Relationship between endorhizal fungal abundance and soil environment

Plant and soil samples from three different treatments including unimpacted, extracted and remediated (Figure 2.1) were assessed in this project. Unimpacted soils analyzed here were naturally overlying the oil sands which have characteristic soil properties for the Athabasca oil sands region. Extracted soils analyzed here were tailing sands produced during the oil extraction process and had some pioneer plants such as *Taraxacum* and *Sonchus* growing on top them.
Remediated soils analyzed here were tailing sands mixed with artificial fertilizer and had plants growing on top of them. The interesting aspect is that plant samples from all of these three different treatments displayed abundant endorhizal fungal colonization (see Section 3.2.1.). Thus, perhaps, the soil microbial (or at least fungal) community appears to be consistent in quantity.

The use of hot water and alkali involved in the oil extraction process was expected to severely deplete soil microflora in tailing sands (extracted soil). However, considering the composition of the original Athabasca oil sands (about 70% inorganic materials including sand, clay, 10% water, and anywhere from 0%-18% oil) there may have been little microflora before extraction. Results showed (see Section 3.2.1.) that the abundance of fungal colonization in dandelion roots was not significantly different between the extracted, remediated and unimpacted treatments. This result does not necessarily mean that microfloras were not destroyed during the extraction process. Perhaps the original abundance of endorhizal fungi was relatively small or it is also possible that the recovery of the soil fungal community was rapid. However, no previous studies have reported whether or not this is the case. It’s also possible that some fungi transmitted with plant seeds were not affected by the extraction process.

Fungal abundance in plants can be affected by diverse factors, such as host-fungus compatibility, multiple fungal species, soil properties, temperature, season and their potential combinations. My study assessed the influence of soil properties, including available soil mineral content, organic matter, hydrophobicity and pH on endorhizal fungal abundance in roots of dandelion in Athabasca oil sands. But due to the limitation of sample size and the issue of unclear collecting sites, the results cannot fully reflect the relationship between fungal abundance and soil properties.
Mycorrhizal fungi, especially AMF, have been shown to play an important role in the acquisition of P and N for host plants. Both P and N are required by mycorrhizal fungi growth (Treseder, 2004), the available P and N concentration are believed to relate to mycorrhizal abundance (Treseder and Allen, 2002). Low soil P and N concentrations has been shown to increase the hyphal growth of mycorrhizal fungi while both P and N fertilization decreases mycorrhizal abundance (Nagahashi et al., 1996; Dodus and Nagahashi, 2000; Treseder and Allen, 2002; Treseder, 2004). According to my soil assay results (see Section 3.1.1.), the available P concentration in soil of unimpacted treatment was higher than the available P concentration in soil of extracted and remediated treatments. Considering the artificial fertilizer added to remediated soil, the difference in P concentration in different soil samples could be due to the fact that the method used might not be sensitive enough to quantify the total available P. The higher available N concentration in soil of remediated treatment relative to extracted and unimpacted treatments may be due to fertilization in the remediated sites. However, there were no significant differences between these treatments in terms of fungal abundance.

Nogueira and Cardoso (2006, 2007) reported that growth of soybean (*Glycine max*) and rangpur lime (*Citrus limonia*) was not affected when the soil P concentration in the soil-sand mixture (three parts of sandy soil (Typic Quartzipsamment) and one part washed sand) is between 50-100 ppm. Plants respond to mycorrhiza positively and negatively when the available soil P concentration is below 50 ppm and above 100 ppm, respectively. High mycorrhizal abundance in all three treatments may result from available soil P concentration in the extracted, remediated and unimpacted soils being lower than 50 ppm (see Section 3.1.1). Also, the difference in available soil P concentration between the three treatments may not be big enough to make a difference in the mycorrhizal colonization rate in host plants from different treatments.
Responses of mycorrhizal abundance to N concentration were reported to be positive (Lussenhop et al., 1998), negative (Klironomos et al., 1998) or not significant (Bethlenfalvay et al., 1999). The inconsistent effect of N concentration may be related to the variation in responses among mycorrhizal groups or among groups of plant and fungi combinations (Treseder and Allen, 2000). Previous research has shown that composition of AMF community is shifted with available soil N concentration. For example, Eom et al., (1999) found the abundance of spores from Gigaspora gigantea and Glomus mosseae increased with N fertilization. In contrast, spores from Entrophospora infrequens declined significantly. The differences in available N concentrations in soil samples of different treatments may change the composition of AMF community but not total abundance of AMF.

The extracted soils were shown to contain the lowest Ca and Na in my assay, while the fungal abundance is still similar to the samples of unimpacted and remediated treatments. Although low soil exchangeable Ca concentration may affect the abundance of AMF colonization in host plants in the way of causing premature root senescence which would have negative effects on AMF sporulation and colonization (Jarstfer et al., 1998), there was no previous research that reported exchangeable Ca and Na concentrations directly controlling the abundance of mycorrhizal fungi. Rodriguez-Echeverria et al., (2008) also did not find any relationship between AMF colonization and concentration of soil exchangeable Ca and Na after they examined AMF abundance and soil properties in six locations of a European coast.

AMF have been observed to concentrate hyphae in organic matter (Hepper and Warner, 1983) and organic matter is important in providing a substrate for the development of some kinds of mycorrhizal fungi (Allen et al., 1993). AMF can form soil structural units (aggregates) through the combined action of AMF hyphae, glomalin (a hyphal wall component) or even
AMF-associated microbes (Rillig *et al*., 2005). Aggregates can provide resistance against erosion and benefit the AMF by reducing erosion-related host plant losses (Rillig, 2004b). Although the organic carbon content of soil samples of the three treatments varied from lowest in the extracted samples and highest in unimpacted samples in my soil assay, the abundance of mycorrhizal colonization are not significantly different among the three treatments (see Tables 3.1.1. and 3.2.1.). Considering the limitation of sample size and the issue of unclear collecting sites, perhaps a larger sample size and corresponding collecting sites will provide more convincing evidence to show the relationship between soil organic matter and mycorrhizal fungal abundance. However, since mycorrhizal fungal abundance results from different factors, the combination of other soil properties should also be considered in addition to organic carbon content.

The occurrence of AMF has been shown to correlate with soil pH in some previous studies (Tadych and Blaszkowski, 2000). But Ursic *et al.* (1997) found that the density of AMF in *Pinus strobus* seedlings was not related to the soil pH. Porter *et al.* (1987) reported that soil pH affects the preference of certain fungi to colonize plant roots. *Acaulospora leavis* is found more in acid soil (~ pH 4.7) than in neutral soil and germination of *Acaulospora leavis* spores requires a pH > 6 (Porter *et al.*, 1987). Three strains of *Glomus* were found to be more adaptable in soil of pH ranging from 5.5-8.4 (Porter *et al.*, 1987). Although in this study the extracted soil samples had a higher pH than the unimpacted and remediated soil samples, there were no apparent differences of mycorrhizal abundance in dandelion roots growing in soil of different pH (see Tables 3.1.1. and 3.2.1.). It is possible that no influence of soil pH on mycorrhizal fungal abundance in this study was found because of the different responses of different mycorrhizal fungi groups to soil pH as well as the dandelion-fungi combination. It is also possible that the pH
difference among these three kinds of soil samples is too minor to influence mycorrhizal fungal abundance.

Many studies have reported that drought stress increases the colonization abundance of mycorrhizal fungi. For example, 43 flowering plants growing on a fallow agricultural site in Germany were examined, 40 of them were heavily infected by AMF in a low soil moisture habitat, while only 29 of them were heavily infected in a comparable but high soil moisture habitat (Kuhn, 1991; Kuhn et al., 1991). In my study, soil samples of remediated and extracted treatments were shown to be relatively hydrophobic (see Figure 3.1.1.). This result implies that the soil moisture may be lower in these two kinds of soil compared to in the unimpacted soil. But there was no apparent difference in mycorrhizal colonization abundance between all three treatments. Spore germination can be increased (Douds and Schenck, 1991), decreased (Tommerup, 1984; Estaun, 1990; Douds and Schenck, 1991) or unaffected (Douds and Schenck, 1991) by soil drying depending on the species. For example, in Douds and Schenck’s study (1991), Acculosporu longula spores were observed to increase germination rate as soil water content increases. Glomus intramildices were observed to decrease germination rate as soil water content increases, while the germination rate of Gigaspora margarita spores were not significantly affected by soil water content (Douds and Schenck, 1991). The similarity of mycorrhizal abundance in my study could be contributed to different compositions of mycorrhizal community in samples from the different treatments. In addition, the influence of soil moisture content on mycorrhizal fungal abundance may be too minor to be observed.

Few studies about the relationship between soil properties and endophytic fungal abundance have been done before. Tian et al. (2003) reported acidic soil is more ideal for the growth and colonization of class 2 endophyte fungi such as Fusarium spp. in rice compared to alkali soil.
Blodgett et al. (2006) showed that the abundance of class 2 endophytic fungi such as *Alternaria tenuissima*-like group and *Fusarium* species associated with *Amaranthus hybridus* were significantly influenced by soil modifications and watering. Rodriguez and Redman (2007) also reported that environmental conditions dictate class 2 endophyte population dynamics in plants co-colonized with endophytes that confer different stress tolerances. However, in my study, the abundance of endophytic fungi (SE) was not significantly different between all three treatments (see Table 3.2.1). For my study, the fungal abundance assay only included fungi colonization in roots, although the distribution of endophytic fungi in below and above ground tissues is different (Tian et al., 2003). The similarity of endophytic fungal abundance may result from the bias of root detection only without considering fungal colonization in the above ground tissue. In addition, whether the SE observed inside of the dandelion roots was class 2 endophyte fungi is not yet clear, as class 2 endophyte fungi isolated from dandelion and sowthistle were not detected in inoculated tomato plants using CLMS (see Section 3.5). However, SE in dandelion roots could be observed using CLMS. If the SE observed were not class 2 endophytes, perhaps factors that affect the abundance of class 2 endophyte fungi may not influence the density of SE. Also, a specific stress (i.e. drought and low mineral content in tailing sands) only increases the population of class 2 endophytic fungi which are adapted to the stress (Rodriguez and Redman, 2007) but may not influence the total endophytic fungal abundance.

All in all, a larger sample size and corresponding accurate collecting site information are necessary for studying the relationship between different endorhizal fungal abundance and soil environment in the future.
4.2. Limitation of methods involved in endophytic fungal recovery and morphological identification

As previously discussed by several authors (Bills and Polishook 1992, 1994; Livsey 1994; Collado et al., 1996; Polishook et al., 1996), the method used for isolation determines the frequency and species distribution of fungi recovered from a certain host. Bills and Polishook (1992) found a mixture of 1% malt extract and 0.2% yeast extract provides the highest species richness for isolations of endophytic fungi from twigs and leaves of *Chamaecyparis thyoides*. Santamara and Diez (2005) reported that incubation in moist chambers resulted in significantly more fungal isolates and much higher species richness compared to isolation on PDA when they tried to recover fungi from *Populus tremula* tissue. Cao *et al.* (2000) reported water agar seemed to be a better medium than PDA for isolating endophytic fungi from *Musa acuminata* leaves and roots. Although PDA has often been cited as a common, unselective medium used for endophytic fungal recovery (Camacho *et al.*, 1997; Rodriguez and Redman, 2008), using PDA may result in underestimating the actual number of endophytic fungal species in plant samples (Santamara and Diez, 2005). Class 2 endophyte fungi may have different nutritional strategies than other groups and rich medium might overwhelm the growth of class 2 endophyte fungi. Therefore, high species richness may result from species of fungi from other endophyte groups.

In my study, 10% PDA at pH 5.0 was the only culture medium used in endophytic fungal recovery. The nutrient concentration and pH at these levels may be more ideal for some strains of endophytic fungi. Therefore, some strains of endophytic fungi cultured on this PDA may have a competitive advantage in terms of growth and resource acquisition. Some fungi may even inhibit the growth of other endophytic fungi through excreting secondary metabolic products, providing another competitive advantage. Involving more kinds of culture medium for isolating
endophytic fungi from plant tissue in future studies may provide a more accurate representation of endophytic fungal species richness.

In this study, plant tissue segments placed on PDA medium were incubated for 5-7 days at 28 °C to allow for the emergence of fungi. Since the growth rate of endophytic fungi can be different, perhaps the incubation time may not be long enough to allow some endophytic fungi to emerge. Future studies may address this issue by prolonging the incubation period. Also, reducing the culture temperature may result with more fungal colonies represented since the temperature of natural soil is lower.

4.3. Possible mechanisms of habitat-specific stress tolerance to host plants conferred by class 2 endophytic fungi

Class 2 endophytic fungi may significantly affect the eco-physiology of different habitats through the rapid adaptation of plants to high-stressed habitats (Rodriguez et al., 2008a). Class 2 endophytic fungi are unique for their ability to confer habitat-specific stress tolerance to host plants (Rodriguez et al., 2008b). Previous research has shown that class 2 endophytic fungi increase host shoot and/or root biomass by the induction of plant hormones by the host or biosynthesis of plant hormones by the fungi (Tudzynski and Sharon, 2002). Class 2 endophytic fungi may confer disease tolerance to host plants by producing secondary metabolites (Schulz et al., 1999), through fungal parasitism (Samuels et al., 2000) and induction of systemic resistance (Vu et al., 2006) or because of an inability of pathogens to compete with endophytic fungi for resources or niche space (Rodriguez et al., 2008a). The cellular mechanisms involved in stress tolerance and growth improvement are not yet clearly known (Rodriguez et al., 2008a). Redman et al. (1999b) reported endophyte-colonized plants activate host defenses more quickly compared
to non-symbiotic plants when exposed to virulent pathogens, while without pathogen exposure, plants colonized with class 2 endophytic fungi do not activate host defenses. This indicated that certain biochemical processes are involved in symbiotically conferred stress tolerance (Rodriguez et al., 2008a).

Some similar plant responses have been found when plants were exposed to heat, drought and salt stress, including increased osmolyte production, altered water relations, production of signaling molecules such as abscisic acid (ABA) and the generation of reactive oxygen species (ROS) (Bohnert et al., 1995; Bray, 1997; Wang et al., 2003; Apel and Hirt, 2004). Rodriguez et al., (2008a) reported there is a strong correlation between ROS susceptibility and stress tolerance in endophytic fungi colonized plants. For example, non-symbiotic plant tissues bleached white and class 2 endophytic fungi (*Fusarium culmorum*) colonized plant tissues remained green after exposed to abiotic stress and then paraquat (a herbicide that induces ROS production and subsequent photobleaching of chlorophyll; Vaughn and Duke, 1983) (Rodriguez et al., 2008a). *Fusarium culmorum* confers salt but not heat tolerance and only protects plants exposed to salt stress from photobleaching but not plants exposed to heat stress. Meanwhile, *Curvularia protuberata*, which confers heat but not salt tolerance, only protects plants exposed to heat stress from photobleaching but not plants exposed to salt stress (Rodriguez et al., 2008b). This result indicates ROS protection in endophytic fungi colonized plants occurs in a habitat-specific manner (Rodriguez and Redman, 2007) and may result in class 2 endophytic fungi conferring stress tolerance to host plants in a habitat-specific manner.

Many previous studies have shown that drought tolerance in plants is usually related to increased osmotic potential (Bohnert et al., 1995; Bray, 1997; Wang et al., 2003). However, Rodriguez et al., (2008b) reported that non-symbiotic plants wilt much earlier (6-10 days) than
class 2 endophytic fungi symbiotic plants even though the osmotic potential does increase in non-symbiotic plants. Symbiotic plants were shown to consume 30-50% less water than non-symbiotic plants (Rodriguez et al., 2008b). In my study, tomato plants inoculated with the class 2 endophyte fungi (*Trichoderma harzianum*) started to wilt at lower average water content in soil compare to axenic tomato plants (see Table 3.4.2). These results suggest that class 2 endophytic fungi conferred drought tolerance through more effective water use instead of correlating with an increase in osmotic potential.

In my study, the class 2 endophyte, a strain of *Trichoderma harzianum*, was isolated from plants growing on tailing sands and found to confer low nutrient and drought tolerance to artificially inoculated host plants (see Section 3.4). Altomare et al. (1999) reported a *Trichoderma harzianum* Rifai 1295-22 (T-22) strain that could solubilize *in vitro* insoluble or sparingly soluble minerals such as MnO₂, metallic zinc, and a rock phosphate (mostly calcium phosphate) through acidification, producing chelating metabolites and redox activity. T-22 has also been shown to enhance nitrogen use efficiency in corn (Harman, 2000). Considering the relatively low nutrient content in tailing sands, the *Trichoderma harzianum* strain isolated from tailing sands samples may increase the available mineral concentration to supply enough essential nutrients for host plant growth. Harman (2000) reported that roots colonized by T-22 reduced the sensitivity of crops to drought stress and resulted in enhanced root development. The *Trichoderma harzianum* strain isolated from tailing sands samples may confer drought tolerance through more effective water use as well as enhance the development of root growth. The latter may increase both of water and nutrient acquisition in symbiont inoculated plants relative to anexic plants that may have a less developed root system. Lynch (Patent No. 7273552) found by providing a compatible rhizosphere, a strain of *T. harzianum* are capable of protecting the plant
as well as being able to detoxify their environment by processing larger amounts of contaminant through higher metabolic rates, the *T. harzianum* isolated from plants growing on tailing sands may confer the tolerance of the host plant to PAH residue in tailing sands in the similar methods.

### 4.4. Molecular identification of *Trichoderma* strain isolated from tailing sands

In my study, ITS1 and ITS2 regions of the *Trichoderma* strain were amplified and sequenced. The sequence information obtained from ITS1 and ITS2 regions were not specific enough to identify the *Trichoderma* strain to species level (see Section 3.8.). Many studies have made heavy use of ITS1 and/or ITS2 (Kuhls *et al.*, 1997; Kindermann *et al.*, 1998; Lieckfeldt *et al.*, 1998; 2001; Dodd *et al.*, 2000) since this gene cluster is present for > 90 copies per genome and thus can be easily amplified. However, using ITS1 and ITS2 becomes discredited in identifying some fungi such as the closely related *Fusarium* and in plants that have been shown to contain paralogous copies of the parts of the rDNA gene cluster (O’Donnell, 1992; Buckler *et al.*, 1997; O’Donnell *et al.*, 1998; Lieckfeldt and Seifert, 2000). ITS was thought to provide poor resolution for closely related species (Druzhinina *et al.*, 2005). In addition, the use of ITS1 and ITS2 provides only poor phylogenetic resolution in some clades, especially *Pachybasium*, which includes *T. hamatum, T. harzianum, T. virens, T. viride and T. koningii* (Kullnig-Gradinger *et al.*, 2002; Chaverri *et al.*, 2003a). The sequence information of ITS1 and ITS2 fragments were submitted to a BLAST and the best match to species was accepted. But the GenBank databases contain many ITS sequences of *Trichoderma* isolates which had been incorrectly identified and thus occur under a false name (Druzhinina and Kubicek, 2005). For example, Kullnig *et al.* (2001) reidentified several strains as *Trichoderma harzianum* that had been reported in the
literature under different names. Therefore, the BLAST result may mislead the identification of *Trichoderma* based on the ITS sequence information.

The translation elongation factor 1 alpha (EF-1α) loci consist of several relatively large and variable introns and exons and in total exceed 2 kb in length. Different fragments of EF-1α were found to be the most promising loci compared to the 11 tested loci/fragments (Druzhinina et al., 2005). Because the sequence information of ITS1 and ITS2 region is not specific enough for *Trichoderma* identification, the sequence information of the section calmodulin gene (cal), actin gene (act) and EF-1α, were performed as a complement of ITS sequence information (Samuels et al., 2006) in Dr. Samuels’ lab. Therefore, in my study the *Trichoderma* strain that conferred a stress tolerance to host plants was identified as *Trichoderma harzianum*. However, *Trichoderma harzianum* is usually a complex of morphologically cryptic species (Chaverri et al., 2003b). Therefore, a future study involving the use of RAPD or AFLP techniques may be needed to confirm whether the isolation I present is a single or multiple *Trichoderma* strain.

4.5. Future research directions

Field trials in a natural tailing sands environment are necessary to confirm and optimize use of the *T. harzianum* strain that confer host plants’ tolerance to tailing sands in small-scaled greenhouse experiments. More host plants such as some weedy species will be used and compared with tomato plants as they are more suitable for practical remediation use in tailing sands.

Some research has shown naphthenic acids (NAs), a kind of toxic organic acid compounds formed during the caustic extraction process, are phytotoxic and cause cell death in the plant root epidermis as well as change the chemistry of parenchyma cells in the root pith.
(Armstrong, 2008). To figure out whether the long-term *T. harzianum* strain treatment can impact the soil environment and naphthenic acid degradation in tailing sands will be a future goal.

The identification and description of other possible endophytes that assist pioneer plant growth in the Athabasca oil sands may also be important in assisting current remediation methods. Based on unique class 2 endophytes which establish habitat-specific symbiosis with their host plants, similar methods as described in this study can be used in other high-stress environments. This could potentially result in transferring the tolerance of the particular abiotic or biotic stress factors conferred by the class 2 endophyte to other host plants.
V. APPENDIX

*Alternaria* spp. isolated from plant samples from unimpacted, extracted and remediated treatments, a *Pseudeurotium* spp. isolated from plant samples from unimpacted treatments and a *Fusarium* spp. isolated from plant samples from remediated treatments were also used to inoculate axenic tomato seedlings. However, all of these fungal strains were harmful to their host tomato plants, resulting in either visibly weakened mature plants or death of the host plant. As the investigation into harmful fungal strains was not the purpose of study, these results were not included and a further analysis was not performed.
VI. REFERENCES


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