Characterization of Fungal Communities Associated to Willow SRIC Plantations in the Canadian Prairies Ecozone Using PCR-Based Molecular Methods

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By

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Dedication

For my parents, my husband, and my lovely little daughter who offered me unconditional love and support throughout the course of this thesis.

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ABSTRACT

Willow (*Salix* spp.*)*, a major source of biomass and renewable fiber production, is one of the best choices for short-rotation intensive culture (SRIC) in Canada. Since fungal communities play important roles in the plant's health status, it is vital to understand their interactions with willows and their roles in the sustainability of SRIC.

In this study, fungal diversity of the above-ground organs (stem/leaf) of healthy and diseased willow plants in western Canadian Prairies were assessed using cultural and PCR-denaturing gradient gel electrophoresis (DGGE) techniques. Comparison of the mycoprofiles within established plantations vs. newly introduced cuttings revealed differences in the fungal communities. *Ascomycota* were mainly isolated, followed by *Basidiomicota* and *Zygomycota*.

Willow genotypes seem have an influence on the abundance of fungal pathogens and disease severity; among them *Charlie* (*Salix alba* x *gladfelteri*) and *SV1* (*S. eriocephala*) cultivars demonstrated superior performances. Photosynthesis measurements and biomass compositions confirmed these findings.

Potentially pathogenic fungi (*Dothioraceae*, *Diaporthaceae*, *Glomeraceae*, and *Pleosporaceae*) dominated in diseased or symptomatic willows, whereas potentially beneficial fungi (*Coniochaetaceae*, *Hypoceraceae*, *Nectriaceae*, *Trichocomaceae*, and *Agaricaceae*) prevailed in healthy plants. *In-vivo* and greenhouse assays showed that inoculation with potentially pathogenic fungi induced leaf necrosis, anthracnose and open cankers. However, suppression of the latter was still possible using fungal antagonists.

Hence, assessment of stem/bark and leaf fungal communities with respect to willow genotypes, cuttings origin, and SRIC location, is useful for the design of an effective management strategy to increase the productivity of the SRIC-biomass systems.

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LIST OF ABBREVIATIONS

BLAST: Basic Local Alignment Search Tool

bp: Base Pairs

BSA: Bovine Serum Albumin

DGGE: Denaturizing Gradient Gel Electrophoresis

DNA: Deoxynucleic Acid

dNTPs: Deoxynuleotide Tri-Phosphate

FISH: Fluorescence *In situ* Hybridization

GenBank: Nucleotide Sequence Database

ITS: Internal Transcript Spacer

NCBI: National Center for Biotechnology Information

OTU: Operational Taxonomical Unite

PCR: Polymerase Chain Reaction

rDNA: Ribosomal Deoxynucleic Acid

rRNA: Ribosomal Ribonucleic Acid

SMCD: Saskatchewan Microbial Collection and Database

SRIC: Short Rotation Intensive Culture

SSU: Small Sub Unit

TE: Tris-EDTA Buffer

UPGMA: Unweighted Pair Group Method with Arithmetic Mean

µl: Micro Litre(10^{-6})

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1. INTRODUCTION

Willow (*Salix* spp.) is one of the best choices for producing woody biomass, bioenergy, and renewable fiber (Picchi et al., 2006). The phytoremediation and CO_2 sequestration capacities of willow have, in the context of the global climate change, redirected governments towards increasing willow trees biomass (Witters et al., 2009). Willow is considered as a good source for generating high yields of biomass with economical and ecological benefits. The willow biomass can decrease the use of fossil fuels and reduce greenhouse effects (Taylor. 2008). Willow trees can be easily propagated from unrooted cuttings. Coppicing willow is made by harvesting willow stems in the dormant period and growing them in the soil again (Smart and Cameron, 2008). Willow has a short breeding cycle (Volk et al., 2004) and is genetically diverse. Its adaptibilty to different climates is responsible for its wide distribution across North America, or in subtropical and tropical regions elsewhere (Kuzovkina and Volk, 2009). High biomass production can be assessed using several components of growth, for example stem diameter, the number of stems, and the wood density. Willow is an efficient producer of biomass as it has high regenerative properties allowing it to produce high yields within a few years. Each willow crop can be harvested successfully up to six or seven times without the need for replanting (Volk et al., 1999; Volk et al., 2004; Kuzovkina and Volk, 2009), and it normally requires less pesticide control compared to other sources of plant biomass (Uellendahl et al., 2008). From their experiments in New Zealand, Hussain et al. (2009) showed that willow cuttings grew faster than poplars by a difference of 10.9 mm in their stem diameter, of 0.55 m in height, and of 35 cm in the canopy diameter at the age of two years (Hussain et al., 2009). The abovement advantages of willows, including *Salix viminalis* and *S.dasyclados*, make them one of the primary sources of renewable energy in different countries (Weih, 2004). For that matter, in North America, *S. eriocephala*, *S. purpurea*, *S. miyabeana*, and *S. sachalinensis* are extensively used for biomass production (Labrecque and Teodorescu, 2005; Kuzovkina and Volk, 2009).

In Canada, research aimed at using biomass as an energy source began in 1978. Since 1980, the government has primarily focused on forest biomass and energy plantations, such as the short-rotation willow plantation technology— the largest nursery work of willow plantations is in Montreal (Picchi et al., 2006).

Fungi play an important role in the functioning of an ecosystem by participating in the decomposition of dead tissues for example (Cornelissen et al., 2001). Unfortunately, among the 1.5 million estimated fungal species, only 74000 (<5%) have been reported (Hawksworth. 2001).

Willow plants may suffer from infections by a wide variety of fungal microorganisms. Research has shown that willow diseases caused by pathogenic fungi became frequent in SRIC after a few growing seasons (Vujanovic and Labrecque, 2002). Fungal diseases cause pathogenic infections on different parts of the willow plant and may lead to reductions in biomass production (Hubes. 1983). There are many factors related to fungal colonization on a particular plant organ, such as plant surface, penetration structure, and the thickness of the cuticle layer. In addition, natural chemical compounds on the host plant can determine the dependency of fungal species to a special part of the host plant and subsequent fungal colonization (Schafer. 1994; Kolattukudy et al., 1995). Pure culture and molecular techniques are used to assess fungal communities in soil and plant tissue samples. Molecular assessments of fungal communities are more accurate due to the omission of a large number of fungi when using culture techniques (Schadt et al., 2003; Vandenkoornhuyse et al., 2002).

The goal of the present study was to isolate and characterize the fungal communities associated with willow plantations and to compare them to original cuttings. Western Canadian plantations were selected to evaluate dominant fungal species present in healthy and diseased above-ground willow samples. Comparison of the obtained mycoprofiles within established plantations and willow cuttings can reveal the differences in fungal biodiversity before introducing cuttings to new plantations. Biomass production and photosynthesis activity were also measured as there is a direct relationship between biomass production and the aerial tissues (leaf functions) of a tree. Functional activities of pathogenic and potentially beneficial fungi isolated from willow samples were tested *in-vitro* and *in-vivo*. Finally, the fungal communities in the same host were evaluated using PCR-DGGE.

The studies encompassed by this project aimed to achieve the following criteria:

1- To assess the fungal biodiversity associated with willow cuttings, as well as healthy and diseased stems/leaves from the SRIC in the Canadian Prairie Ecozone regions using PCR-based molecular methods;

2- To compare biomass and photosynthetic activity between healthy and diseased plants using stem diameter and fluorometric analyses;

3- To evaluate the functional effects (beneficial and pathogenic) of isolated fungi from willow samples *in-vitro* and *in-planta*; and

4- To compare differences and/or similarities of the biodiversity in fungal communities between cultural and non-cultural (DGGE) methods.

This document begins with a review of important aspects of willow Short Rotation Intensive Culture (SRIC), determination of fungal biodiversity, microbial ecology techniques, and application of DGGE fingerprinting method for profiling fungal communities in Chapter 1. Chapter 2 describes the fungal biodiversity in willow stem and leaf samples and their prevalence in sampling sites. This chapter also explores the same goal in willow cuttings, as we believe that cuttings can be the origin of many fungal isolates before they are transferred to plantation sites. Chapter 3 reports the effects of the fungal biodiversity on willow biomass production and photosynthesis activity. Finally, Chapter 4 is dedicated to studying the effects of potentially beneficial and pathogenic fungi isolated from the above-ground tissues of willow.

2. LITERATURE REVIEW

2.1. Importance of Willow (*Salix* spp.) for Producing Biomass and Bioenergy

Willow is one of the best woody species in terms of potential for biomass production in both agricultural lands and forests. Under Short Rotation Intensive Culture (SRIC) many European countries and the US grow this crop successfully and produce high crop densities. With climate change and greenhouse effect concerns, due to industrialization and associated environmental and air pollution, as well as increasing demands for fuels due to energy demands of the growing world population, humans are putting increasing effort into finding better sources of renewable energy (Radmanesh et al., 2006,Konecsni 2010). Thus, willow has become a good alternative in providing biomass energy (Fennica, 2010). Willow is a good choice for generating high yields of biomass because of four key traits. Firstly, willow can be easily propagated from unrooted cuttings. In fact, willow cuttings are produced by harvesting willow stems in the dormant period and planting them in the soil during the spring for producing more plants. Willow crops can be harvested successfully up to six or seven times without replanting because of their ability to resprout after harvesting. Secondly, willow is genetically diverse, permitting it to adapt to different climates such as alpine, continental, tropical, and subtropical regions. Thirdly, willow has a high growth rate and relatively short breeding cycle. The amount of heat produced from a dry ton is similar to several other hardwood plants such as poplar (Aylott, 2008; Row, 2009).This last characteristic is especially important given that the main use of willow as a short rotation crop is that of a renewable source of energy, facilitating decreasing use of fossil fuels and reducing greenhouse effects (Reijnders. 2006; Taylor. 2008).

Willow can retain solar energy very efficiently. Because of this, producing energy from willow crops would require less input energy units in comparison to other bioenergy plants (Koski and Dickmann, 1992) such as poplar, switch grass (McKendry. 2002), topinambours, sunflower (Jasinskas et al., 2008), sweet sorghum or sugar cane (Nguyen and Prince, 1996). The use of intensive willow plantations would temporarily decrease the amount of CO_2 in the atmosphere (Toivonen and Tahvanainen, 1998).

Furthermore, willow crops are very useful for wastewater management (Aasamaa, 2010), landscaping and landfills (Börjesson and Berndes, 2006). Soil water content is important for plant productivity (Guidi, W. 2010). In addition, willow plantations can have extra environmental benefits such as phytoremediation (Mleczek, 2010; Abhilash, 2010). In addition,

over the past few years, healthy stems of willow cuttings (usually *S. viminalis*) have been planted to create "green wall structures" (GWS), as vegetative barriers in urban areas. GWS are most frequently established alongside highways in highly polluted urban areas in order to reduce noise and air pollution. Replacement of concrete barriers with willow stems can improve acoustic quality and add more beauty to the surrounding areas. Moreover, it can result in better air quality through removal of excess air pollution and providing more oxygen (Vujanovic and Labrecque, 2008). GWS could also decrease maintenance costs as willow would not require re-planting and is capable of self-repair. Furthermore, willow short rotation cropping is expected to have positive effects on economy and regional employment for farmers (Toivonen and Tahvanainen, 1998). Two main problems with willow crops are the cost of cultivation machinery and the development or identification of suitable clones resistant to pests (diseases and insects). In order to achieve maximum productivity, the density of willow plantation should be about 10,000 - 20,000 cuttings per hectare (Hytönen et al., 1995). In Canada, a cultivation system where plants are grown densely and stems are coppiced and harvested frequently, following a 2 to 4 years harvest cycle, seems favourable for the development and spread of diseases (Labrecque and Teodorescu, 2005). Also, different clones vary in their susceptibility to various microbial groups. Pathogenic and beneficial microorganisms could differently impact clone biomass production in relation with management practices for soil, species and cultivar, agro-technical measures, planting density, the harvest cycle, and pest control (Stolarski, 2010). The aim of this study is therefore to discover microbial communities associated with each willow clone or taxonomic unit grown in Canadian Prairies (AB, MB, SK) under accepted management practices.

Roughly 330 to 500 species of *Salix* are recognized so far (Argus, 1997). Willow species are distributed over the Northern hemisphere but there are a few species native to the Southern hemisphere as well (Kuzovkina and Quigley, 2005). Genus *Salix* is divided into five subgenera: *Salix, Protitea, Longifoliae, Chamaetia,* and *Vetrix* (Table 2-1). The subgenus *Salix* as a tree-type species has many things in common with poplus including large size, leaf stalk and arborescent growth. *Salix alba* (white willow), *S. lucida* (shining willow) and *S. babylonica* (weeping willow) are the most popular and famous representatives of subgenus *Salix* (Kuzovkina and Volk, 2009; Smart and Cameron, 2008). Common native North American black willow (*S. nigra*) and peach leaf willow (*S. amygdaloides*) are derived from subgenus *Protitea*. Subgenus *Longifoliae* includes sandbar willow (*S. interior*) which is a new world species with the ability to

propagate through root shoots. Among this genus, *Vetrix* is probably the most abundant subgenus because it contains more species. Some of them include heart-leave willow (*S. exiocephala*), goat willow (*S. caprea*), American pussy willow (*S. discolor*) and basket willow (*S. viminalis*). Finally, subgenus *Chamaetia* includes low-growing plant species such as snow bed willow (*S. herbacea*) which are well adapted to the harsh conditions in the arctic zone (Argus, 2007). *Salix dasyclados* and *S. viminalis* are the species that are used the most biomass production in Europe (Weih, 2004). However, *S. alba, S. purpurea, S. miyabeana, S. eriocephala* and *S. sachalinensis* have been already naturalized in North America and can be good choices for biomass production in North America (Smart et al., 2008; Tharakan et al., 2005).

In Europe and North America, *S. viminalis* has been used for biomass production in SRIC. Experiment with this species has shown that growing multiple species or clones in a willow plantation leads to the highest biomass productivity while minimizing the risk of disease and pest infection (Mitsui, 2010).

Table 2-1. Willow (*Salix* spp.) genotypes used in SRIC bioenergy plantations, as potential hosts for mycobiota (Smart and Cameron, 2008).

Subgenus	**Section**	**Species**
Vetrix	*Cinerea* (grey willow)	*S. discolor*
		S. caprea
	Viminella (basket willow)	*S. viminalis*
		S. schwerinii
		S. sacramensis
		S. desyclados
	Cordatae (sanddune willow / furry willow)	*S. eriocephala*
	Geyeriane	*S. petiolans*
	Fulvae	*S. bebbiana*
	Helix	*S. purpurea*
		S. miyabeana
		S. integra
		S. sachowensis
		S. koryanagi
	Daphnella	*S. daphnoides*
	Hastatae	*S. cordata*
Longifoliae	*Longifoliae*	*S. interior*
		S. exigua
Salix	*Llumboldtianae*	*S. amygdaloides*
		S. nigra
	Amygdalinae	*S. triandra*
	Salix	*S. alba*
	Subalbac	*S. babylonica*
	Salicaster	*S. lucida*

2.2. Fungal Biodiversity

2.2.1. Determination of Fungal Biodiversity

In previous centuries, biodiversity studies have focused mainly on macro organisms such as plants and animals. However, plants and animals have evolved relatively recently; microorganisms dominate the tree of life and were the dominant inhabitants of the earth for more than 3.7 billion years (Zak, 1994). Another important reason for study of microbial biodiversity is that life is dependent on microbial functional diversity (Zak, 1994). Fungal organisms can live in a wide variety of oxygen, temperature, pH and other environmental conditions. Fungi have a prominent role in maintaining the environment through decomposition processes. For this reason, any change in their communities reflects on and impacts the ecosystem (Leben, 1965). Many fungal organisms live on other creatures such as humans, animals and plants, where they play diverse roles as pathogens, saprophytes, parasites, biocontrol agents or symbionts. Biodiversity studies allow us to gain insight into both the distribution patterns and functional roles of fungi. The term diversity refers to qualitative variation among microorganisms (Øvreås, 2000). Biodiversity incorporates two concepts: 1) the total number of species presents, termed species richness, and 2) the distribution of the species, termed species evenness. Because of difficulties related to taxonomy and classification, using Operational Taxonomic Unit (OTU) is a good method to describe different phenotypes in biodiversity studies.

Fungal diversity research is not only important for basic science, but to understand the correlations between fungal community structure and functions. Human influences on the environment, such as pollution and chemical applications can affect fungal diversity. Also, agriculture can have a major impact on fungal communities in comparison to un-cultivated lands, resulting in either increases or decreases in abundance and biodiversity (Daniell, 2001; Buckley, 2001). Unfortunately, the study of biodiversity and function is limited by methodological limitations and taxonomic difficulties. Moreover, the exact functional roles of many fungi are still unknown, as are their susceptibilities or tolerances to abiotic or biotic stresses. It is generally assumed that a diverse microbial community can be more resilient to stress conditions and is more capable of adapting to environmental changes. However, our knowledge related to molecular techniques is developing step by step, facilitating a growing understanding of the complexity of plant-fungal organisms' interactions (Klironomos, 2003). In addition, biological

controls of plant pathogenic fungi are important in future studies. Furthermore, understanding of the structure of fungal communities and their diversity patterns can lead to a deeper comprehension of pathogen-antagonist interactions. To overcome the cultivability limitations, a series of molecular methods have been chosen. These include amplification of ribosomal DNA (rDNA) and sequencing and denaturing gradient gel electrophoresis (DGGE) of rDNA. Such molecular techniques were expected to clarify the fungal community structure in the environmental willow samples and permit a comparison with a set of cultured fungi from the previous year plantations. In addition, in our study, the application of molecular finger printing method (DGGE) for evaluating fungal diversity in willow above-ground tissues are described. It is expected that molecular techniques can reveal a realistic perspective of fungal species richness, diversity and distribution**.**

Fungal organisms play very important roles in ecosystems including marine, fresh water, soil, living plants and dead materials. They could be pathogenic to other living organisms such as plants and animals or could form symbiotic relationships with the plants (Smith and Read, 1997). Examples of symbiotic associations include mycorrhizae and fungal endophytes (Garg and Chandel, 2011). Furthermore, fungi play a critical role in mineralization of carbon through the decomposition process. They also help plants to obtain essential nutrients via underground mycelial networks. It is estimated that the total number of fungal species on earth is around 1.5 million (Hawksworth, 2002; Hawksworth. 1991). This estimate is based on data of known fungal species that were studied previously and data gathered from plant host studies. Although the exact total number of known fungal species is unclear, it is thought to fall in the range of 72,000 to 100,000 species. So far, we can say that whatever we know about fungal diversity is less than 5% of the total. In other words, we only know 1 in 20 of existing fungal species. So, the big challenge of finding the unknown 1.43 million fungal species remains to be addressed. (Hawksworth, 2002; Hawksworth and Rossman, 1997).

Fungi can be found in almost all ecosystems. It has even been suggested that a wide variety of fungi are present in deep-sea environments at 10, 000 m below the surface (Nagano, 2010). Fungi can play a powerful role in the environment through nutrient cycling. Yet, science has not discovered all of the fungal species and their roles in the environment. Many ecologists are facing difficulties related to culture methods. Because of these difficulties, most of them prefer to use molecular methods which permit analysis of samples isolated from natural

environments through fingerprinting, without passing through culture-based approaches. Usually, when a specific plant (such as willow) becomes the focus of attention it is highly probably that many fungal species will be found. For instance, Kohlmeyer et al. (1998) found that the maritime rush, *Juncus roemerianus*, which grows on east coast of the US, is the host of numerous fungal species. Among them, 20 fungal cultures were identified as new species, including 8 new genera and 1 new family (Kohlmeyer et al., 1998). This example shows that plants can be a good habitat for many unknown fungal fauna and flora. One of the major challenges is that no fungus-free plant species was used as a control in those data or the clear plant species without fungi were not included in the study. Besides, with these collected data, we cannot predict how many new species are on leaves of a tree or how many of them are similar between both healthy and diseased plant organs. For this reason, in a well-designed study, it is wise to consider supported data such as above mentioned questions (Hawksworth and Rossman, 1997). As evidence, there are many fungal pathogens of willow above-ground tissues, well-adapted to North American ecosystems which are not studied in Canadian bioenergy plantations (Table 2-2).

Recently, there has been a worldwide effort made to protect plants through limitations of xenobiotics usage. Examples of xenobiotics include herbicides and pesticides used to control plant pests and weeds (Lemanczyk and Sadowski, 2002). Looking for environmental friendly alternative methods such as beneficial fungi has become a matter of interest in many research centers and universities.

Table 2-2. Characteristics and distribution of major fungal pathogens on willow in North America according to Hubes (1983).

Fungal Taxon	Found in willow	Disease Note	Distribution	Host	Substrate
Glomerella cingulata	stem/leaf	Anthracnose of stems and leaves, dieback, root rot, leaf spot, blossom rot, fruit rot	Cosmopolitan	Multiple genera in multiple families	—
Valsa malicola isolate 256 *Valsa malicola*	stem stem	Associated with dieback	Asia, Europe, North America	*Malus* spp. and other Rosaceae	Dead or dying twigs
Anthostomella conorum	stem/leaf	Associated with leaf spots	Widespread	Multiple genera in multiple families	—
Cytospora chrysosperma strain xsd08012	stem	Bark canker of *Populus* spp. (poplars), also causes necrosis of *Salix* spp. (willows)	Africa (Morocco), Asia, Australia, New Zealand, Europe, North America (Canada, Mexico), South America (Chile).	Mostly *Populus* spp. and *Salix* spp. (Salicaceae), but also other hardwoods	Bark of twigs.
Valsa sordida	stem/leaf	Bark canker of *Populus* spp. (poplars), also causes necrosis of *Salix* spp. (willows). Often associated with the pathogenic fungus *Leucostoma niveum*	Africa (Morocco), Asia, Australia, New Zealand, Europe, North America (Canada, Mexico), South America (Chile)	Mostly *Populus* spp. and *Salix* spp. (Salicaceae), but also other hardwoods	Bark of living and dead twigs
Phoma medicaginis	stem	Black stem	Cosmopolitan	*Medicago* spp., *Melilotus* spp. (Fabaceae) and other plants	Seed-borne, stems
Gibberella avenacea	leaf	Blight, head blight of wheat, rots of fruits, stems, and roots	Cosmopolitan, most common in temperate regions	Multiple genera in multiple families	
Cytospora pruinosa	leaf	Canker, dieback	North America, Europe	*Fraxinus* spp. and other Oleaceae	Wood, branches and twigs
Leucostoma niveum	stem	Colonizes bark of weakened trees, cauing necrosis or canker of twigs and branches	Asia, Europe, North America (USA, Canada).	Principal host: *Populus* spp.. Also other Salicaceae, Betulaceae, Rosaceae	Bark of dead or dying, attached or fallen twigs

Nectria haematococca	stem/leaf	Fruit rot, stem rot	Cosmopolitan	Trees of various plant families	Bark, decorticated wood, herbaceous tissue, fruits, fungal sporocarps, roots, soil
Leptosphaerulina trifolii	leaf	Leaf spot	Cosmopolitan	Multiple genera in multiple families	Leaves
Alternaria brassicae	leaf	Leaf spot	Cosmopolitan	Brassicaceae.	Living leaves
Alternaria triticina	leaf	Leaf spot, blight	Africa, Asia, North America (Mexico).	Triticum spp., e.g. Triticum aestivum (Poaceae)	Fruit, leaves, seeds, soil.
Cladosporium variabile	leaf	Leaf spot, mold	Cosmopolitan	Spinach, *Spinacia oleracea* (Chenopodiaceae)	Leaves. Seed-borne
Botrytis byssoidea	leaf	Neck rot, damping off.	cosmopolitan	*Allium* spp. (Liliaceae)	—
Phoma glomerata	stem/leaf	Opportunistic pathogen. Found in association with blights, leaf spots, fruit rots.	cosmopolitan	Various plant genera	Soil, dead seed coats, animal tissues, and inorganic material (e.g. paint, chemical products) (plurivorous)
Valsa ambiens	stem	Probably a weak parasite	Cosmopolitan	Woody angiosperms from multiple plant families	Wood, dying twigs
Chaetomium globosum	stem	Saprobe	Cosmopolitan	Multiple genera in multiple families	—
Nigrospora oryzae	stem/leaf	Saprophyte and weak parasite; cob and stalk rot of maize, wheat	North America, Europe	Multiple plant families	Plant parts, air, soil
Sclerotinia sclerotiorum	leaf/stem	*Sclerotinia* disease, *Sclerotinia* wilt, *Sclerotinia* rot, stem blight, head rot	Cosmopolitan	Multiple plant families	Root and above-ground plant parts
Apiospora montagnei	leaf	Secondary invader or saprophyte	Cosmopolitan	Multiple plant families	Plurivorous. Living /dead plant , air, animals, soil

Monilinia laxa	leaf	Wilt, blight, canker, brown fruit rot. Overwinters in mummified fruits	North America (Pacific Northwest), South America, Europe, Asia, Australia, Africa (South Africa).	Rosaceae, primarily *Prunus* spp., also apples (*Malus* spp.), pears (*Pyrus* spp.) and other Pomoideae	Blossoms, shoots, twigs, fruits
Leucostoma persoonii	stem/leaf	Wound pathogen, causing mass wilting of branches and dieback of young trees, canker	Africa, Asia, Europe, North America, South America (Brazil), Australia, New Zealand	Principal hosts: Rosaceae; also *Alnus* (Betulaceae)	Dead or dying, attached or fallen twigs

2.2.2. Environmental Nucleic Acid (DNA) Extraction

Nucleic acid contents can be extracted from environmental samples such as leaves and stems using DNA extraction kits and also other protocols (Griffits et al., 2000). Achieved nucleic acids from this method are mixed, consisting of DNA from fungi, plants and/or other microorganisms. It is clear that the efficiency of this technique depends on species presence, environmental substrate and the extraction method. There are several approaches for analyzing the total amount of DNA. The required information about the fungal community defines which approach should be chosen for analyzing the extracted DNA. However cost, time and the number of samples also are important factors in this kind of research. At present PCR-based molecular techniques is the most universal method for studying fungal communities. The advantage of this method is selectively amplifying the fungal DNA with the help of fungal specific primers. A number of specific primers have been developed which allow separation of fungal sequences from a mixed DNA sample (Kennedy and Clipson, 2003). In many cases, these primers not only can discriminate fungi from other kingdoms, but also they are able to differentiate among different fungal groups and species level. This method is helpful in case of overlapping fungal sequences with other eukaryotic organisms (Anderson et al., 2003).

For a successful PCR amplification from the environmental samples, it is better to purify the nucleic acids. Usually during nucleic acid extraction some PCR-inhibitor compounds such as phenolic compounds, humic acids, and some polysaccharides can be co-precipitated with the nucleic acids. For removing these impurities we can dilute our DNA content or use selective detergents (for instance, cetyl-trimethyl-ammonium bromide-CTAB) to skip over the polysaccharides. Also using clean-up columns such as silica-sephadex G-200 or hydroxyapatite can be helpful for omitting the inhibitory contaminants. Also, in case of high volume

environmental samples, using calcium chloride density centrifugation will be helpful (Mitchell and Zuccaro, 2006).

2.2.3. Choice of Target Gene in Molecular Diversity Assessment

Since its introduction in 1986, PCR has become one of the most important tools in the field of molecular biology. Many PCR-based molecular techniques have been developed to better understand microorganism community structures. Generally, DNA provides a template for PCR amplification of target genes with the universal (non-discriminative) primers to amplify target sequences in the samples. Choosing the target gene for sequencing is very important and requires previous knowledge of the gene fungal sequences. In order to differentiate based on nucleotide sequences, it is crucial to employ a discriminative PCR method. For this reason, selection of a gene marker is a very important step for molecular assessment in microorganism's communities. This genetic marker must be informative, potentially variable between the microorganisms of interest, present in all selected subgroups and possess a conserved region in which primer annealing sites can be located. In summary, an effective target gene for research on functional diversity should express phylogenetic differences among functional groups of genes (Justé et al., 2008).

Many PCR primers which can amplify fungal DNA from a variety of taxonomical groups have been described; however few of them were designed for environmental samples. For this purpose, a PCR primer must be highly specific to fungi, because in environmental samples fungal DNA is less abundant than that of plants or other eukaryotes (White et al., 1990). Since last decade of 20^{th} century, the internal transcribed spacer (ITS) of the rRNA gene cluster has been utilized for investigating fungal species from environmental samples (Grades and Bruns, 1996; Jonsson et al., 1999 a&b).

2.3. Microbial Ecology Techniques

2.3.1. Microbial Community Analysis Techniques and Microbial Ecology

Analyzing the diversity of microorganisms, such as fungi, require reliable isolation and classification of species. Thanks to the molecular microbiology we know that less than 1% of the

microorganisms in the environment are cultivable (Robe, 2003), necessitating techniques to isolate and characterize the uncultivable portion of microbial biodiversity (Countway, 2005). These new approaches rely on analysis of the very small pieces of ribosomal RNA genes which could be categories in two important groups: molecular probes (such as fluorescent *in situ* hybridization (FISH) and DNA fragment analysis (such as denaturing gel gradient eletrophoresis (DGGE)). Analysis via molecular methods requires previous knowledge of the targeted community structure. In contrast, DNA fragment analysis does not necessary use such previous knowledge, but does require extraction of DNA and subsequent amplification with universal primers. However, both approaches describe the dominant components in the microbial ecology, meaning they cannot detect subdominant species which could be important in environmental adaptation (Galada, 2005).

Molecular techniques offer faster and more accurate diagnosis of plant pathogenic fungal inoculation or disease (McCartney, 2003). Microbial communities can normally be investigated based on the three basic properties of circumscription, identification and qualification. It is obvious that none of the present molecular techniques is able to draw a complete picture of microbial biodiversity. However, in general, there are two kinds of PCR-based approaches which are routinely used in molecular microbiology. One of them is using universal primers for different taxonomic groups, which can create a mix of amplicons and go through a range of analyzing methods. In addition, group-specific PCR reactions are also available for detecting specificity of the genes of interest (Justé et al., 2008). Different molecular methods have been used in recent decades to study and characterize microbial ecosystems. All of them involve multi-steps procedures, which include community structure, identification, isolation and finally qualification (Justé et al., 2008). Culture-dependent methods have been used for several decades to investigate microbial biodiversity in different environmental habitats. Different general and selective media have been utilized to isolate various microorganisms (Bull, 2004). Physiological factors such as pH and nutrient materials availability play an important role for culturing different microorganisms (Zinder and Salyers, 2001). However, conventional cultural methods will not allow us to isolate and characterize many fungal organisms from their natural environments. Many fungal organisms cannot be grown in the laboratory, illustrating the difficulty of separating fungal biomass from the environment. Development in molecular microbiology helps many laboratory researches to analyze 18S rDNA and DNA data from

natural ecosystems (Schabereiter-Gurtner et al., 2001; Schabereiter - Gurtner et al., 2001). However, developing and choosing PCR primers which target fungal DNA without co-amplifying the non-fungal DNA is a major challenge. There are a lot of primers that can amplify fungal DNAs in many taxonomic communities, but few of them are suitable for use in mixed environmental samples. Use of poorly chosen primers could lead to inaccurate calculations of fungal biodiversity (White et al., 1990). In 1993, two scientists, Gardes and Bruns, designed the primers known as ITS1-F and ITS4-B. These primers amplify fungal ITS regions without co-amplification in mixed environmental samples (Gardes and Bruns, 1993). Several other primers have been designed for isolating fungal phylum, such as *Ascomycota, Basidiomycota, Chytridiomycota* and *Zygomycota* through direct DNA extraction from mixed environmental soil samples (Smit et al., 1999; Borneman and Hartin, 2000). This problem is due to similarity between the 18S rRNA gene sequence of fungi and other eukaryotic organisms. This fact provided incentive to design a new generation of primers named EF4/EF3 and EF4/fung5 targeting fungal 18s rDNA (Smit et al., 1999). However, there is a lot of debate about the newly designed fungal primers (18s rDNA) (Smit et al., 1999; Borneman and Hartin, 2000). For instance, EF4/EF3 and EF4/fung5 primer have been shown to amplify fungal 18S rDNA from wheat rhizoids (Smit et al., 1999) and to amplify unwanted non-fungal DNAs (Bachmann and Specialist, 2002).

2.3.2. PCR and DGGE in Microbial Ecology

During the past two decades, several successful approaches have been developed in the field of applied microbiology. These molecular methods allow us to overcome the limitations related to culture-dependant methods (such as speed, taxonomic resolution and reproducibility) and improve microbial diversity studies (Muyzer and Smalla, 1998). DGGE was invented by Leonard Lerman and his colleague Stuart Fischer at the State University of New York, Albany (Fischer and Lerman, 1983). Within about five years of the introduction of these approaches both DGGE and TGGE became some of the most rapidly growing molecular tools in molecular and ecological microbiology (Muyzer and Smalla, 1998). Since microbial ecology is the study of living microorganisms in their natural ecosystems; we have to study them in their natural habitats. The best approach is to extract them from their natural environment and then characterize them. Denaturing Gradient Gel Electrophoresis (DGGE) and Temperature Gradient

Gel Electrophoresis (TGGE) are of the practical molecular methods which were developed to study biodiversity in environmental samples. Both DGGE and TGGE are useful for analyzing nucleic acids and, in some cases, for separating proteins (Fischer and Lerman, 1979). These methods have become more and more known in the domains of molecular microbiology and microbial ecology (Giraffa and Naviani, 2001).

In 1993 Gerard Muyzer first described the coding of small ribosomal pieces of rDNA. Later, DGGE was widely used in microbial ecology. After extracting DNA either from the colony grown on media or directly from the plants, PCR amplification can be done. Usually general 18S rRNA primer sets will be employed to target and amplify eukaryotic micro-organisms. In contrast, for bacteria and archaea most commonly used primer set for PCR amplification is 16S rRNA. Because these amplification products have the same length, they cannot be separated from each other through traditional gel electrophoresis. This problem can be solved by using the denaturing gradient gel electrophoresis approach and then mysteries of biodiversity among microbial communities can be solved. With the technique of DGGE, we are able to distinguish between different members of the microbial community and hence estimate relative abundances (Anderson et al., 2003). DGGE has been used in the field of microbial ecology to analyze the biodiversity of environmental samples. With the help of this technique, we can study microbial dynamics in different species. Because of its simplicity and reproducibility DGGE has found its place in molecular microbial laboratories very fast (Sanders. 2002).

In addition, T/DGGE can be used as a fingerprinting approach to assess stability of microbial diversities (Masco et al., 2005; de Souza et al., 2004). DGGE has been used effectively in estimating and evaluating biodiversity among prokaryotic and eukaryotic microorganisms in environmental samples (Jeewon and Hyde, 2007). Traditionally, the existence of microorganisms in different environmental samples has been studied by the culture-based methods. However, it is now accepted that these methods are usually less feasible for characterizing minor populations or microbial communities, which require selective enrichment or special nutritional requirement. Furthermore, stress or weakness can affect some microbial cells that could increase their needs and necessitate special recovery condition for cultivation. Because of these limitations, we are forced to develop culture-independent methods such as molecular techniques. These relatively new techniques are usually based on PCR-amplification and detection of nucleotides related to

the most dominant microorganisms (Yang et al., 2001). In comparison with other molecular methods, TGGE and DGGE approaches are faster, more specific and more sensitive (Justé et al., 2008). DGGE is a common method in molecular biology and for evaluating community structures. Also, DGGE has been used in medical researches as a more rapid tool for detecting mutants such as single nucleotide polymorphisms (SNPs) (Gadanho and Sampaio, 2006). In DGGE, DNA fragments of the same length, but different nucleotide composition, are separated according to the movement of a denatured double-stranded DNA in polyacrylamide gel. Denaturizing is caused by either a temperature gradient or chemicals such as formamide and urea. It is expected that we can isolate close to 100 percent of DNA sequences up to 500 bp in length attached to GC-clamp (5′-CGCCCCCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC-3′) in PCR amplification (Anderson et al., 2003; Sheffield et al., 1989). In this technique, a short fragment of Guanines and Cytosines bases will be added to DNA sequences at the end of 5' (Anderson et al., 2003). Length of the clamp varies from 40 to 45 nucleotides (Manter and Vivanco, 2007; Justé et al., 2008). In order to obtain good DNA bands in a denaturing gel, it is important to have enough DNA. This means PCR is a crucial step prior to DGGE analysis. Under increased temperature or chemical concentration, double stranded DNA starts to melt, separate and migrate in the gel. This method is one of the best techniques for analyzing small single pieces of DNA made up of approximately 400 base pairs (Malosso et al., 2006; Manter and Vivanco, 2007).

Most dominant fragments of DNA will make a single band in a narrow range of denaturing gradient. This allows us to search for differences in the DNA sequences. For instance, different fragments of DNA will separate in different locations and melted fragments will stop in different positions in the gel. This can provide us information on which sequences are having higher similarity to each other. In this comparison, researchers can detect DNA fragments with mutation(s). Also, by running two different DNA sequences on the gradient gel, these samples can show their differences from each other side-by-side. This provides good capability for recognizing biodiversity in different microbial communities (Manter and Vivanco, 2007).

Examples of TGGE/DGGE approaches used in microbial ecology include study of the microorganisms communities (Kumaraswamy et al., 2005), and changes in those communities (Ward et al., 1997; Short and Suttle, 2002), monitoring the microorganisms' enrichment and

growth (Li et al., 2006), comparing different extracted DNAs (Heuer and Smalla, 1997), clone libraries (Felske et al., 1999), and PCR determination (Keohavong and Thilly, 1989). Vainio et al. (2000) used DGGE techniques to find wood-inhabiting fungi and compare the phytotypes isolated from natural (environmental) samples with those isolated from the same host in different depths of the wood samples (Vainio and Hantula, 2000). Nikolcheva et al. (2003) tried to use this method (DGGE) on fungal communities associated with living leaves. They examined fungal diversity on decaying leaves in freshwater associated with different plants such as oak, red maple and beech. Their most prominent result in this study was the highest rate of fungal diversity during one week after submerging the leaves in water. Later, Nikolcheva (Nikolcheva et al., 2005) studied fungal biodiversity with the same technique in first stage of leave decaying in different hosts (oak, maple leaf and linden).

Nowadays, DGGE is one of the best techniques for understanding microbial biodiversity (Muyzer et al., 1993). In DGGE, each taxa will show a particular band according to the electrophoresis mobility of DNA molecules, which can then be used to infer biodiversity (Yergeau et al. 2005). Moreover, DGGE/TGGE methods are affordable and yield easy to interpret result. In addition, we can excise individual bands of interest from the gel for re-amplification and re-sequencing (Baker et al., 2004).

3. FUNGAL BIODIVERSITY IN WILLOW ABOVE-GROUND TISSUES GROWN UNDER SHORT ROTATION INTENSIVE CULTURE (SRIC)

3.1. Abstract

Prediction of fungal species in tree propagation materials is important before introducing them to new geographical regions. In Canada, willow cuttings are imported from foreign countries for the establishment of Short Rotation Intensive Culture (SRIC) as one of the best choices in biomass production for bioenergy. Profiling the mycodiversity of willow cuttings is important in maintaining high density willow plantations for the purpose of biomass production. In this study, microbiological and molecular techniques were used to characterize fungal communities associated with healthy willow cuttings imported in to Canada from the United States. A total of 82 fungal taxa from asymptomatic cuttings were isolated and identified. *Ascomycota* was the predominant phylum, although some *Basidiomycota* (*Agaricales* and *Tremellales)* were also detected. The most abundant fungal taxa belong to *Hypocreales,* whereas *Kabatiella microsticta* was the most dominant species. Our results support findings that some potentially pathogenic fungal taxa of willow plants (*Glomeraceae*, *Diaporthaceae* and *Venturiaceae*) may originate from the cuttings. PCR-denaturing gradient gel electrophoresis was successfully standardized and used to identify non-cultivable fungal species in environmental samples of willow cuttings. *Lecythophora* spp. was the most frequently observed species based on the results of the DGGE method. These results were discussed in light of the potential fungal influence on willow in SRIC and the current strategy of preventing exotic fungal pathogens from entering Canada. Furthermore, analysis of fungal communities between healthy and diseased plants allows discrimination of pathogenic versus biocontrol/beneficial fungi on willow. For this reason, healthy and diseased willow stem and leaf samples were collected from different plantations in Saskatchewan, Alberta, and Manitoba. A total of 106 fungal taxa from healthy and diseased stem and leaf samples were isolated. As in cuttings, *Ascomycota* was the most predominant fungal phylum in stem/leaf samples. The most abundant fungi belonged to *Pleosporales,* and *Diaporthales*. *Alternaria* spp. and *Cytospora* spp. were the most dominant in all sampling sites. Based on the DGGE method, *Alternaria* spp., *Cytospora* spp., and *Davidiella* spp. were the most dominant species inhabiting willow above-ground tissues. PCR-denaturing gradient gel electrophoresis was successfully standardized and used to identify non-cultivable

fungal species in willow environmental samples. These results may influence management strategies in willow SRIC.

3.2. Introduction

Biological invasion of exotic plant pathogens is a serious threat to the agroforestry sector, especially for willow in Short Rotation Intensive Culture (SRIC) (*Salix* spp.), as providing rapid biomass production for bioenergy has become very popular in Canada. Non-indigenous or allochtonous fungal diseases can adversely affect the growth, survival, and reproduction performance of willow (Mooney and Hobbs, 2000). A considerable proportion of worldwide national budgets are already invested into controlling native and exotic pathogens and in preventing crop damage annually by applying quarantine measures and chemical pesticides (Pimentel, 2000). The non-indigenous fungus *Ophiostoma ulmi* (*Ceratocystis*) is a well known example of a devastating fungal tree pathogen causing "Dutch elm disease" throughout North America (Wingfield, 2010). Over the past decades, the entry of the fungus *Cryphonectria parasitica,* the cause of 'chestnut blight' in North American forests, led to the extinction of many native plant species in the early part of the previous century (Merkle, 2011). Some fungal pathogens have a relatively narrow host range (e.g. *Puccinia* spp. causes rust diseases) (Schulze-Lefert, 2011), whereas some others such as *Pythium* spp. have the capacity to infect a wide variety of trees/hosts (Stewart, 2010). Additionally, it is noteworthy that fungal pathogens with a low pathogenic significance in one geographic region often create huge epidemic issues once established in a new geographical region. As evidence, *Nectria coccinea,* the fungus that causes Beech Bark Disease, was imported from Europe to the eastern coast of North America in 1929 (Houston, 1994). Recently, the new invasive fungus *Phytophthora ramorum,* associated with "sudden oak death", and capable of infecting several other plant species in North America, is believed to have originated from leaf spot and dieback of European rhododendron plants (California Oak Mortality Task Force, 2001). The truth is that the effects of non-indigenous plant pathogens on new agriculture, forestry or agro-forestry systems could be permanent. Many environmental as well as economical factors can prevent or control the pathways involving the arrival of new plant pathogens in natural ecosystems and tree plantations. It is further recognized that standard quarantine or sanitary management practices are at the forefront of the control measures, since various plant tissues (cuttings, seeds, fruits, and wood) and products are moved

widely over the world. There are some mandatory prohibitions against known pests, as well as regulations regarding the importation of plant/tree propagation materials that are potential carriers of those pests. In the agro-forestry sector, a serious problem could appear, associated with the importation of tree cuttings for the establishment of new biomass plantations across North America. Currently, there is no available information about the fungal invasiveness potential associated with cuttings, since the asymptomatic cuttings are allowed to enter new geographical regions without any restriction. Many willow cuttings used in North America originated from northern Europe (e.g. Sweden) and eastern Asia (e.g. Japan). Importation of willow cuttings for the development of bioenergy plantations and establishment of SRIC in Canada is a good example of introducing propagative materials in the absence of supporting information about the national system of plant protection. The fact is that prediction of the invasion level requires more in-depth scientific information, and at the same time, effective monitoring and processing of any existing information. Therefore, regulating the new arrival of apparently healthy cuttings for microbial pests, in particularly potential tree fungal pathogens, remains a challenging responsibility because of the involvement of global trade transporting tree reproductive material among countries and geographical regions without strict control of fungi in apparently healthy plant material.

Despite some preliminary understanding about the roles of fungi in Canadian willow ecosystems (Hubes 1983; Vujanovic et al. 1998; Vujanovic and Labrecque 2002 and 2008), there is limited information available about the relationships between fungal biodiversity of imported healthy cuttings, as initial material for establishment of willow plantations, and subsequent fungal effects on three productivity or biomass produced under SRIC.

Profiling fungal diversity associated with willow above-ground tissues (especially cuttings) is important in maintaining high density willow plantations. Several studies have investigated the effects of fungal communities on willow tissues as fungal pathogens seem to be a serious threat for the success of willow biomass production. Adair et al. (2006) pointed out that about 45 fungal species can attack different willow species in the Northern Hemisphere. Understanding the relationship between fungal taxonomic richness and function according to willow genotypes has also been directed in new research (Zak and Visser, 1996; Hawksworth. 2001; Mueller and Schmit, 2007).

Some of the most invasive pathogenic fungal strains reported on willow barks include: *Cladosporium* sp., *Cytospora* sp., *Epicoccum* sp., *Valsa* sp., *Venturia* sp., *Glomerella* sp., and *Leucostoma* sp. (Harman and Zealand, 2004; Vujanovic and Labrecque 2002). Furthermore, *Ascomycota* fungi with different identified phenotypic genera such as *Cryptodiaporthe*, *Drepanopeziza*, and *Glomerella* have been identified as being responsible for serious diseases in basket willow (Vujanovic and Labrecque, 2008). These fungal pathogens can cause various pathological effects, such as blights, cankers, leaf and bark spots, and rust. There exist extensive and diversified fungal communities on willow tissues, especially on aerial parts of the plant. Many of them could be potential biocontrol agents to protect the plant from invasion of other fungal pathogens (Harman and Zealand, 2004). Hence, it is important to choose a disease-resistant plant for producing higher amounts of natural sources of cuttings for willow biomass production. Profiling fungal communities in willow cuttings can also lend ideas about the origins and alterations of fungal inhabitants in willow organs. Fungal assessment and characterization at the species level is usually based on a combination of cultural, phenotypic, and molecular-based methods (ITS rDNA sequence analyses) (Vujanovic and Labrecque, 2008). Determination of fungal biodiversity using a PCR-DGGE method has many key advantages for studying fungal biodiversity (Corredor, 2011). The diversity detectable by the DGGE method depends on the proportion of species abundance in mixed fungal community samples. DGGE can be used simultaneously with conventional laboratory methods to achieve a more precise understanding of fungal communities.

3.3. Objectives and Hypothesis

The hypotheses underlying this study were:

1- Willow cuttings have different fungal community compositions when compared to willow above-ground tissues (leaf and stem) grown in SRIC.

2- Fungal biodiversity and community structures vary among certain geographic regions, health status, plant organs, original cuttings, and different clones.

3- A more diverse fungal community is associated with diseased plants than healthy plants.

4- Healthy plants contain a higher number of beneficial fungal taxa which can be considered as biocontrol agents to restrict colonization of pathogens.

Therefore, the objectives of this study were to: *i)* profile the fungal diversity in willow aerial parts (stem/leaf and cuttings) grown under SRIC, *ii)* use cultivation and DGGE methods to develop a standard marker for DGGE molecular approach for a rapid and simple screening of willow tissues.

3.4. Materials and Methods

3.4.1. Study Design and Sampling

In the present study, a culture-based method and DGGE technique were used to identify fungal communities associated with willow above-ground samples. The samples were separated into two different categories; 1) Healthy class (Healthy versus Diseased), and 2) plant organs (Bark or Stem versus Leaf). Equally healthy-looking plants and plants showing symptoms of diseased (limited growth, stem canker, leaf necrosis) were randomly selected within each of the plantations in 2007 and 2008 (Tables 3-1 and 3-2). Plantations were located on different soil types for the development of willows for agro-forestry and bioenergy in Canada (Table 3-2). In this study, only clone *Hotel* was investigated to compare fungal communities in the geographical locations. In addition, fungal communities associated with stem/leaf of seven different willow clones were investigated. A standard design was used to distribute willows with three row beds at 60 by 60 cm tree spacing with 200 cm between beds, and an average of 150 plants per bed (15625 plants per ha) (Volk et al., 2004). In addition, healthy cutting samples obtained from the United States (Sunny ESF-Tully, NY, 42°47’30”N, 76°07’30”W) were used to identify their fungal communities before introducing them to Canadian plantations (Table 3-2).

The samples were stored individually in separate paper bags and transported back to the Applied Microbiology laboratories at the University of Saskatchewan and quickly stored at 4 °C before the isolation of fungi or culturing procedures.

Table 3-1. Geography and location of the SRIC-willow sampling sites

Location	Site	Coordinates	Year of establishment	Clone	Species of origin
Saskatchewan (Sk)	Saskatoon (Sk1)	UTM13UE467155.03, N5872599.0	2005	*Hotel*	*S. purpurea*
	University (Sk2)	UTM 12U E0389931.8 N5776381.7	2006	*Charlie* *Hotel* *India* *SV1* *Juliet* *SX64* *SX61*	*S. alba X glatfelteri* *S.purpurea* *S. dasyclados* *S. eriocephala* *S. dasyclado* *S. sachalinensis* *S. miyabeana*
Alberta (Ab)	Edmonton	UTM 12U E0330943, N5921366	2006 2005	*Hotel*	*S. purpurea*
Manitoba (Mb)	Portage La Prairie	UTM14U0559416, N5534076	2005	*Hotel*	*S. purpurea*
Sunny-ESF(Tully)	US, NY	424730N, 760730W		9980-005,9879, 9970-036, *SX64*, *SV1*, 9870-23, S365, 99201-007, S25, *SX61*, 9871-31	

Plantations were established in three Canadian provinces (Ab, Sk, Mb). Ab; Alberta, Sk1; plantation situated in Saskatoon, Sk2; University of Saskatchewan plantation including seven clones, and Mb; Manitoba. Sunny-ESF (NY) is the origin of cutting samples consisted of 11 clones.

Table 3-2. Soil characteristics of the SRIC-willow sampling sites

Location	**Edmonton, Ab**	**Saskatoon, SK1**	**Portage la Prairie, Mb**	**University of Saskatchewan (Sk2)**
Name	Ellerslie Research Station	Saskatoon Berry-Barn	Arendse Farm	University plantation
Texture	Clay	Clay overlaying Sandy Loam	Clay Loam overlaying Sandy Loam	Heavy clay
pH	5.5-7.5	8.1-8.5	8.1-8.5	6.5-7.5
%C	3.9-8.1	2.3-3.9	1.6-4.7	2.0-2.6
% N	not available	0.19-0.28	0.25-0.35	0.18-0.30
Mean annual precipitation (mm)*	459.6	348.3	514.5	348.3
Past Crop	Cereals	Cereals	Shallots	Cereals
Year of sampling	2007, 2008	2007	2008	2007, 2008

- Climate average for the last 30 years Environment Canada (2010).

3.4.2. Data collection and Identification of Fungal Taxa using Culture-based method

The collected samples were surface-sterilized by submerging them in 95% ethanol (10 sec), autoclaved distilled water (10 sec), 5% sodium hypochlorite (commercial bleach, 2 min) and finally again with autoclaved distilled water for 2 min. They were then left on filter paper inside the laminar flow hood until completely dry (Mavragani. 2008). These samples were either used in the culture-dependent method or the direct DNA extraction method (environmental samples). After being cleaned and sterilized, the samples **(**around 1 cm segments) were placed onto plates of potato dextrose agar medium (PDA) (Difco, Detroit, Mich.) supplemented with antibiotics (streptomycin sulphate 500 µl/L plus kanamycin sulphate 200 µl/L, Sigma-Aldrich, St.Louis, MO, USA). Five plates containing five fragments were prepared for each sample. Assay plates were incubated in the dark at room temperature for one week. Pure cultures were established using standard microbiological procedures. Pure cultures were regrouped into Operational Taxonomical Unites (OTU) according to their morphotype such as appearance, colour and texture (phenotypic approach). All isolates have been deposited in the Saskatchewan Microbial Collection and Database under accession numbers SMCD2500- 2605 (for stem/bark and leaf samples) and SMCD2606- 2687 (for cuttings). For each OTU, DNA extraction and sequencing were performed as described below. Extraction of DNA was made from fresh pure cultures using the DNeasy Plant Mini Kit (Qiagen Inc., Mississauga, ON) following the manufacturer's instructions.

The internal transcribed spacer (ITS) rDNA was used for amplifying target region of fungal genomic DNAs using fungal specific primers ITS1-F/ITSF4 (Gardes, 1993; White et al., 1990), LS1/ LR5 (Hausner et al., 1993; Rehner and Samuels, 1995), and NS1/NS6 (Simon et al., 1992; White et al., 1990). The conditions used for PCR amplifications were the same as published by the respective authors. The PCR products were purified using a DNA purification kit (QIA quick PCR purification kit, QIAgen Inc., Mississauga, ON, Canada). Later, all the purified DNA samples were sent to be sequenced at the PBI, Plant Biotechnology Institute, Saskatoon, SK. Subsequently, similarity analyses were carried out using Basic Local Alignment Search Tool (BLAST) available from NCBI (http://www.ncbi.nlm.nih.gov). Each fungal taxon was taxonomically classified according to the Index Fungorum Database (www.indexfungorum.org).

3.4.3. Data collection and Identification of Fungal Taxa using PCR-DGGE Procedure

3.4.3.1. PCR Primers Design and DGGE Standard Markers

Internal transcribed spacer (ITS rDNA) sequences of nine different fungal species recovered from the culture-based method were aligned using Clustal-W multiple sequence alignment program version 1.8 (Thompson et al., 1999). Sequences were visually checked for regions with homologies. Optimal sequences for forward and reverse primers were designed according to the primer design guideline (Mitsuhashi, 1996) with highly conserved DNA (< 500 bp; optimal base pair length for DGGE) and containing a high site-specific variation. A GC-clamp (5′-CGCCCCCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC-3′) was attached to the 5' end of the reverse primer to prevent complete denaturation and enhance separation during DGGE analysis (Sheffield et al., 1989). Primers were commercially synthesized (Invitrogen, CA, USA). In addition, the specificity and feasibility of the new designed primers were assessed by PCR amplification of ten randomly chosen DNA samples extracted from fungal isolates belonging to *Ascomycota* and *Basidiomycota.*

An easier, alternative method to the sequencing of DGGE bands is the comparison of the bands with certain reference patterns, to allow for better discrimination. Therefore, prior to running DGGE analyses, two reference standards' PCR-DGGE profiles were generated using DNA extracted and amplified from the pure cultures. These fungal species were expected to occur in the DGGE profile. In order to construct a molecular ladder, six reference fungal isolates as beneficial fungi were used for developing a standard marker for DGGE (Figure 3-1, A) as well as seven reference pathogenic fungi (Figure 3-1, B). The priority for choosing these isolates was based upon their abundance and function.

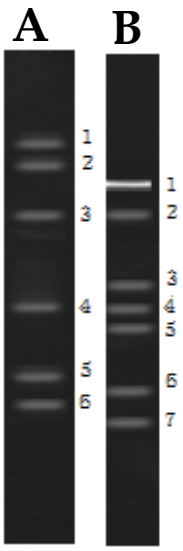


Figure 3-1. DGGE standard ITS rDNA markers designed for screening willow above-ground tissues samples; (A) Potentially beneficial fungal species including 1) *Cadophora melinii*, 2) *Lecythophora* sp., 3) *Kabatiella microsticta*, 4) *Coprinellus* sp., 5) *Microdiplodia* sp., and 6) *Cladosporium cladosporioides*. (B) Potentially pathogenic fungal species including 1) *Davidiella macrospora*, 2) *Arthrinium sacchari*, 3) *Nigrospora oryzae*, 4) *Glomerella cingulata*, 5) *Alternaria* sp., 6) *Leucostoma persoonii*, and 7) *Cytospora chrysosperma*.

3.4.3.2. DGGE

Following DNA extraction using 200 mg tissue samples, a polymerase chain reaction (PCR) was performed. The achieved amplicons were diluted (1:10) and used as templates for secondary PCR amplification using designed primers. Positive secondary PCR amplicons were amplified using a GC-clamp primer prior to running DGGE gels (Sheffield et al., 1989; Simon et al., 1992; Kowalchuk et al., 1997). After visualization on agarose gel to confirm the expected band sizes, DGGE gels were set with the aid of a C.B.S. Scientific Gradient Delivery System (model GM-40 2001). The gradient gels (18×16 cm^2 with 0.75 mm thickness) contained 7% (wt/vol) of acrylamide/bis-acrylamide (37.5:1 acrylamide; Sigma) gels with a range of 30-70% denaturant gradient. For preparing each gel, we used 11.5 ml each from 30% and 70% acrylamide solutions (Mavragani, 2008), 5 µl TEMED, and 80 µl Ammonium persulphate (APS), and then allowed the gel to solidify for about one hour ahead of loading samples. 20 µl PCR products plus 5 µl DGGE loading dye were loaded on the DGGE gels. Molecular markers derived from a known fungal species were loaded on both sides of the gel to facilitate band-to-band comparisons. The gels were run at 80 V for 16-18 hours in 1X TAE buffer (PH=8.0) at a constant temperature of 60°C. Finally, the gels were stained with SYBR Green for 30 minutes. The gel photographs were viewed by the computer program Gel Doc-IT imaging system (UVP Inc., CA).

When sequence information was required (bands migrating to an unknown position), bands were excised under UV trans-illumination (UVP, CA, USA-Model LM-26E), using sterile blades. The gel pieces were solubilized in 30 µl of TE buffer at room temperature overnight and centrifuged at 10,000 x *g* for 2 minutes. 2 µl of the DNA solution was used for PCR re-amplification using the same primer (without GC-clamp). DNA samples were then purified and sent for sequencing (PBI, Saskatoon, Canada). Similarity of the achieved 18S rDNA sequences were compared with deposited sequences in GenBank (NCBI).

3.4.4. Data and Statistical Analysis

Fungal biodiversity indices were calculated using the Shannon-Wiener index and the species richness (Mavragani, 2008). Shannon diversity index (H´) was calculated using the formula: $H' = - \Sigma P_i \times \log_{10} (P_i)$. P*i* is the relative abundance of each fungal species calculated as the proportion of a given individual species of the total number of individuals in the fungal

community: $P_i = n_i / N$ (N is the total number of all individuals, n_i is the number of individual in species and ″i″ is the abundance of species). S (species richness) was also calculated as the total number of species – 1/ Log of total number of individuals. In addition, frequencies of the isolated fungi were calculated as follows:

% frequency = (Number of observations in which a species appeared / Total number of observations) X 100 (Gautam, 2009).

Cluster analyses were carried out by NTSYS pc, Numerical Taxonomy System, version 2.2 (Rohlf, 2001) for providing a dendrogram of different fungal communities. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering method was used to display the variability of strains in healthy and diseased samples. Cluster analysis was performed on DGGE banding patterns by scoring each band in the profiles as absent (0) or present (1) (Liang et al., 1998). Correspondence analyses were carried out by SYSTAT analysis (SYSTAT 10, Inc.) to confirm the origin of fungal cultures collected from different places or regions. Phylogenetic trees were analyzed by MEGA 4.1 software program (Tamura, 2007) to show the inferred evolutionary relationships among various fungal cultures.

3.5. Results

3.5.1. Fungal Diversity in Willow Cuttings

Ascomycota was the dominant phylum among asymptomatic and healthy cutting tissues using the culture-based method, PCR amplification, and DGGE technique (Tables 3-3 and 3-4). The most abundant fungal taxa found in willow cuttings belonged to the order *Hypocreales* using both techniques. *Dothioraceae* and *Coniochaetaceae* were the most frequent fungal families using culture-based method and DGGE, respectively. According to the frequency of fungal distribution, *Kabatiella microsticta* (16.4%) was the most abundant fungal species based on pure culture analysis while *Lecythophora* spp. (20%), was the most frequent isolates using the DGGE method. PCR-DGGE analysis showed individual banding patterns with a number of distinguishable bands ranging from 1 to 7 represented as different OTUs numbers. Sequencing of all bands excised from the DGGE gels showed similar percentages in range of 83% to 100%.

Table 3-3. Fungal diversity in willow cuttings based on OTUs-phenotypic characteristics and rDNA sequences

Description	Total number of cultures isolated	OTU	SMCD	DNA sequence	Associated accession number	Percent identity
Phylum Ascomycota						
Incertae sedis, Amorphothecaceae						
Amorphotheca resinae strain DAOM 170427	1	106.1	2606	LS	EU030280.1	98%
Incertae sedis, Glomerellaceae						
Colletotrichum circinans	1	102.3	2619	ITS	EU400140.1	95%
Glomerella acutata isolate 202	2	109.3	2648	LS	FJ588238.1	99%
Glomerella cingulata strain 1	4	101.2	2649	ITS	AJ301952.1	99%
Glomerella cingulata strain 2	1	105.3	2650	ITS	AB278185.1	96%
Glomerella cingulata strain 3	1	109.4	2651	ITS	AJ301952.1	98%
Dothideales, Dothioraceae						
Kabatiella microsticta	1	101.4	2655	ITS	EU167608.1	99%
Kabatiella microsticta CBS 342.66 strain 1	2	122.1	2656	ITS	EU167608.1	98%
Kabatiella microsticta CBS 342.66 strain 2	14	103.1	2657	ITS	EU167608.1	99%
Kabatiella microsticta CBS 342.66 strain 3	1	117.3	2658	ITS	EU167608.1	95%
Kabatiella microsticta CBS 342.66 strain 4	1	119.5	2659	ITS	EU167608.1	96%
Capnodiales, Davidiellaceae						
Cladosporium cladosporioides strain STE-U 3683	1	126.3	2617	ITS	AY251074.2	99%
Cladosporium sp. B5B	2	104.5	2618	ITS	EF432298.1	99%
Coniochaetales, Coniochaetaceae						
Coniochaeta ligniaria	1	116.4	2620	ITS	AY198390.1	93%
Coniochaeta velutina strain Jong108	1	120.5	2621	LS	FJ167402.1	100%
Coniochaeta velutina strain UAMH 10912	1	114.3	2622	LS	EU999180.1	100%
Lecythophora luteoviridis	1	103.2	2660	ITS	DQ404354.1	96%
Lecythophora luteoviridis strain 64	2	112.4	2661	LS	DQ404354.1	97%
Lecythophora sp. olrim22	1	116.3	2662	ITS	AY781229.1	97%
Lecythophora sp. UBCtra1453C strain1	1	106.4	2663	ITS	AY219880.1	98%
Lecythophora sp. UBCtra1453C strain2	3	119.3	2664	ITS	AY219880.1	96%
Lecythophora sp. UBCtra1453C strain3	2	128.4	2665	ITS	AY219880.1	97%
Eurotiales, Trichocomaceae						
Penicillium commune isolate wb193	1	117.5	2668	ITS	AF455527.1	99%
Penicillium lanosum strain P11.4	1	122.2	2669	ITS	EU833224.1	99%

Penicillium sp. 269B	1	117.2	2670	ITS	GQ120969.1	94%
Helotiales, Incertae sedis						
Cadophora luteo-olivacea isolate PhiK5II strain 1	1	126.4	2609	ITS	GQ214536.1	94%
Cadophora luteo-olivacea isolate PhiK5II strain 2	1	124.2	2610	ITS	GQ214536.1	96%
Cadophora luteo-olivacea isolate PhiK5II strain 3	1	124.3	2611	ITS	GQ214536.1	98%
Cadophora luteo-olivacea strain 18	1	109.2	2612	ITS	DQ404348.1	91%
Cadophora malorum isolate PhiK3II strain 1	2	118.1	2613	ITS	FJ486274.1	98%
Cadophora malorum isolate PhiK3II strain 2	1	118.6	2614	ITS	FJ486274.1	95%
Cadophora melinii 435 strain 1	1	115.5	2615	ITS	DQ404351.1	96%
Cadophora melinii 435 strain 2	2	119.4	2616	ITS	DQ404351.1	97%
Helotiales sp. B54J3	1	118.5	2652	ITS	EF093147.1	93%
Phialocephala sp. L48 isolate L48 strain 1	1	112.2	2673	ITS	FJ903362.1	100%
Phialocephala sp. L48 isolate L48 strain 2	1	117.1	2674	ITS	FJ903362.1	99%
Phoma cava isolate olrim63	1	129.1	2675	ITS	AY354263.1	98%
Phoma glomerata	2	104.1	2676	ITS	AB470828.1	98%
Hypocreales, Bionectriaceae						
Bionectria ochroleuca strain xsd08089	1	113.2	2608	ITS	FJ478131.1	99%
Hypocreales, Hypocreaceae						
Hypocrea viridescens CIB T10 strain 1	2	107.3	2653	ITS	AJ279483.1	99%
Hypocrea viridescens CIB T10 strain 2	1	108.2	2654	ITS	AJ279483.1	98%
Hypocreales, Nectriaceae						
Cosmospora vilior strain 1	1	103.3	2625	ITS	FJ824628.1	95%
Cosmospora vilior strain 2	1	129.6	2626	ITS	AY618257.1	79%
Fusarium larvarum var. *rubrum* strain F-155,597	2	108.3	2638	ITS	EU860068.1	95%
Gibberella avenacea	1	101.3	2639	ITS	EU255802.1	98%
Gibberella avenacea isolate FA01	1	111.5	2640	ITS	EU255791.1	98%
Gibberella avenacea isolate FA06	1	109.1	2641	ITS	EU255796.1	99%
Gibberella avenacea isolate FA11 strain 1	1	102.2	2642	ITS	EU255801.1	99%
Gibberella avenacea isolate FA11 strain 2	1	122.3	2643	ITS	EU255801.1	98%
Gibberella avenacea isolate FA12 strain 1	2	116.2	2644	ITS	EU255802.1	99%
Gibberella avenacea isolate FA12 strain 2	1	120.1	2645	ITS	EU255802.1	98%
Gibberella avenacea isolate FA13	1	125.2	2646	ITS	EU255803.1	99%
Gibberella sp. UFMGCB_536	1	110.1	2647	ITS	FJ466715.1	98%
Nectria vilior	3	106.2	2666	ITS	U57673.1	97%
Neonectria ramulariae	1	118.4	2667	ITS	AJ279446.1	96%
Hypocreales, Ophiocordycipitaceae						
Ophiocordyceps sinensis	1	104.3	2630	ITS	FN386283.1	100%

Pleosporales, Leptosphaeriaceae						
Coniothyrium sp. ICMP 17485	1	125.1	2623	ITS	EU770235.1	97%
Pleosporales, Phaeosphaeriaceae						
Phaeosphaeria herpotrichoides isolate UFMGCB 2623	1	115.2	2671	ITS	FJ911873.1	74%
Pleosporales, Pleosporaceae						
Uncultured Pleosporaceae	1	126.6	2687	ITS	AJ879683.1	99%
Pleosporales, Venturiaceae						
Protoventuria alpina strain CBS 140.83		111.3	2681	LS	EU035444.1	99%
Sordariales, Cephalothecaceae						
Phialemonium dimorphosporum	1	105.2	2672	ITS	AB278185.1	93%
Sordariales, Lasiosphaeriaceae						
Podospora appendiculata-IFO 8549 strain 1	1	120.6	2677	ITS	AY999126.1	93%
Podospora appendiculata-IFO 8549 strain 2	2	127.5	2678	ITS	AY999126.1	92%
Podospora appendiculata-IFO 8549 strain 3	1	128.2	2679	ITS	AY999126.1	98%
Podospora didyma strain CBS 232.78	1	127.3	2680	ITS	AY999127.1	90%
Xylariales, Amphisphaeriaceae						
Truncatella angustata	1	120.4	2685	ITS	AF377300.1	98%
Xylariales, Diatrypaceae						
Diaporthe eres strain xsd08090	1	118.3	2627	ITS	FJ478132.1	98%
Xylariales, Xylariaceae						
Rosellinia nectrioides strain CBS 449.89	1	111.4	2682	ITS	FJ175181.1	98%
Phylum Basidiomycota						
Agaricales, Psathyrellaceae						
Coprinellus sp.	1	122.4	2624	ITS	EU436684.1	99%
Tremellales, Tremellaceae						
Tremella foliacea strain CBS 6969	1	129.4	2684	ITS	AF444431.1	93%

● OUT (Operational Taxonomic Unit) numbers from 101.2 to 129.6.

Pure cultures were grouped into operational taxonomic units based on their morphology and with the aid of microscopy for doubtful cultures (Vujanovic et al., 2007).

Table 3-4. Fungal diversity in willow cuttings based on DGGE analyses

Description	Total number of cultures isolated	OTU	DNA sequence	Associated accession number	Percent identity
Phylum Ascomycota					
Capnodiales, Davidiellaceae					
Cladosporium sp. strain 1	2	J4.2	ITS	EF432298.1	99%
Cladosporium sp. strain 2	2	J8.3	ITS	GU212394.1	99%
Coniochaetales, Coniochaetaceae					
Lecythophora luteoviridis	3	J3.1	ITS	DQ404354.1	96%
Lecythophora sp. strain 1	1	J6.1	ITS	GU062289.1	99%
Lecythophora sp. strain 2	1	J11.3	ITS	GU067748.1	99%
Dothideales, Dothioraceae					
Aureobasidium pullulans	1	J11.2	ITS	FR667988.1	98%
Kabatiella microsticta	3	J1.2	ITS	EU167608.1	99%
Helotiales, Incertae sedis					
Cadophora luteo-olivacea strain 7R38-4	1	J4.3	ITS	GU212374.1	83%
Cadophora melinii	1	J8.1	ITS	DQ404351.1	96%
Hypocreales, Hypocreaceae					
Trichoderma atroviride strain SGSGf39	1	J3.2	ITS	EU715667.1	99%
Hypocreales, Nectriaceae					
Cosmospora vilior isolate olrim557	1	J11.4	ITS	AY805574.1	95%
Fusarium acuminatum strain NRRL 54218	1	J10.1	ITS	HM068326.1	100%
Fusarium larvarum	2	J11.1	ITS	FN868469.1	96%
Incertae sedis, Glomerellaceae					
Glomerella acutata	1	J8.2	ITS	AM991136.1	98%
Glomerella cingulata	3	J1.1	ITS	AJ301952.1	99%
Phylum Basidiomycota					
Agaricales, Psathyrellaceae					
Coprinellus sp.	1	J9.1	ITS	EU436684.1	99%

● OUT: Operational Taxonomic Unit

According to the Hubes checklist, 25.9% and 16% of isolated fungal taxa were potentially pathogenic to willow trees (Hubes, 1983) using cultural and DGGE methods, respectively. These fungal pathogens belong to different taxa, including *Gibberella* spp., *Nectria vilior, Fusarium larvarum*, *Neonectria ramulariae*, *Phoma* spp., *Glomerella* spp., *Colletotrichum circinans*, and *Protoventuria alpina* (Tables 3-3 and 3-4).

Basidiomycota, including *Agaricales* and *Tremellales*, comprised less than 2% of total isolated fungi. Potentially beneficial fungal isolates included mostly *Basidiomycota*, orders *Agaricales* (*Coprinellus* spp., 0.9%) and *Tremellales* (*Tremella foliacea,* 0.9%) as well as fungal endophytes (8.6%).

The Shannon diversity index (H´) indicated a high diversity in the fungal community, equal to 1.72. Furthermore, the species richness was high (37.6). Sequence alignment analysis confirmed many of the relationships in the distance tree (Figure 3-2). *Amorphothecaceae* and *Glomerellaceae* families were in a sister clade with *Coniochaetaceae*. *Venturiaceae* family is a separate branch in this tree. The close relationship between these species supports the morphological similarities of their teleomorphs and anamorphs.

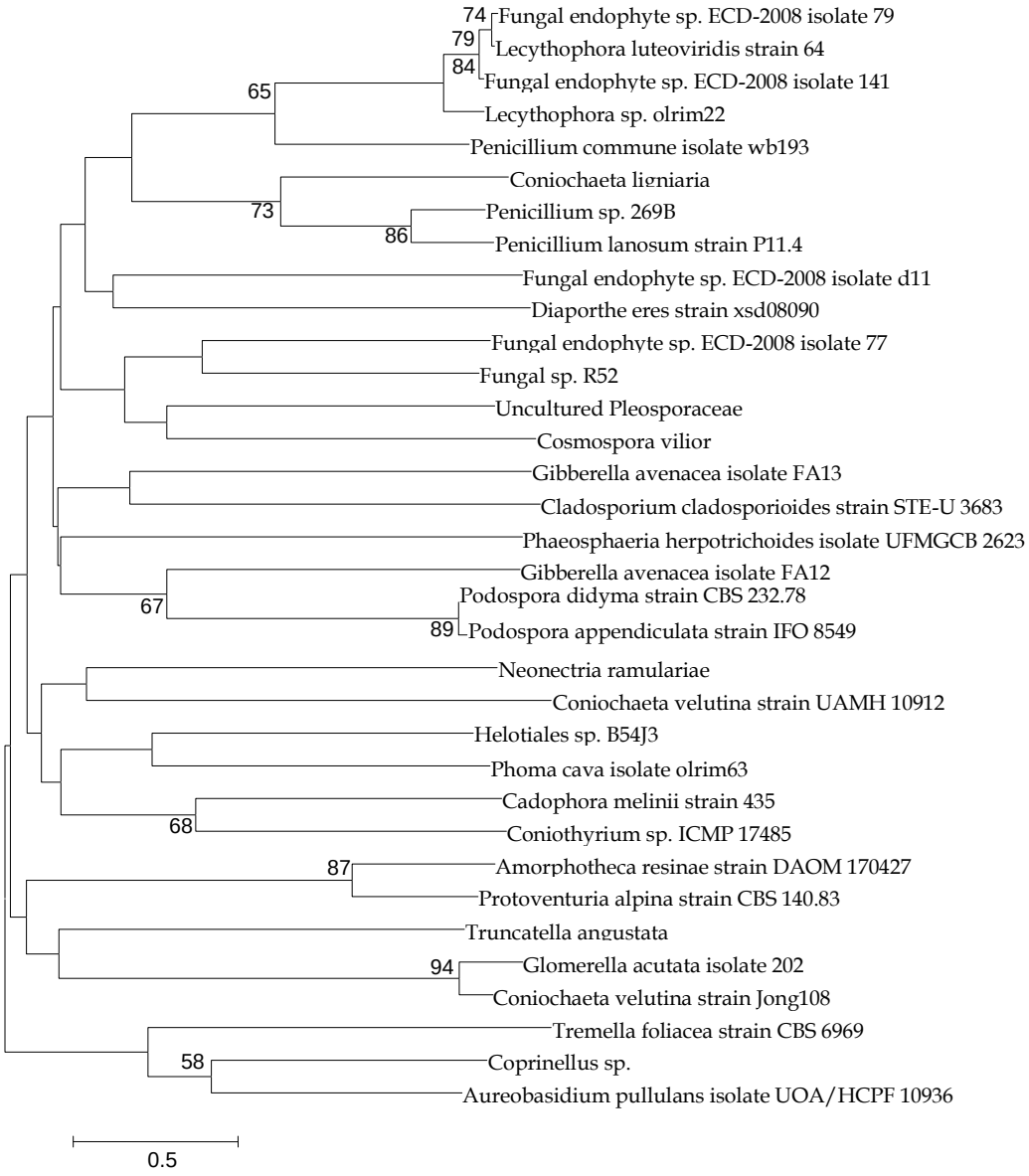


Figure 3-2. Bootstrap tree (maximum parsimony analysis) showing the relationships among isolated fungal species from willow cuttings constructed on the basis of their internal transcribed spacer rDNA sequences. Bootstrap values were obtained from 500 replications. The numbers above the clades are the bootstrap values. See table 3-3 for the description of the fungal isolates.

3.5.2. Fungal Diversity in Willow Stem/Bark and Leaf

The results of this part of the study showed that 106 fungal taxa out of 208 OTUs of phylogenetic groups were isolated using the culture-based method and DGGE technique. *Ascomycota* was the most abundant fungal isolate in stem/leaf samples using both techniques. The majority of the isolated fungi based on the cultural method belonged to *Pleosporales* (37.7%), followed by *Diaporthales* (28.9%) whereas *Capnodiales* (28.8%) and *Diaporthales* (27.5%) were the dominant group using the DGGE method. The evaluation of prevalence indicated that *Cytospora* spp. were the most predominant taxa based on both methods (Figure 3-3).

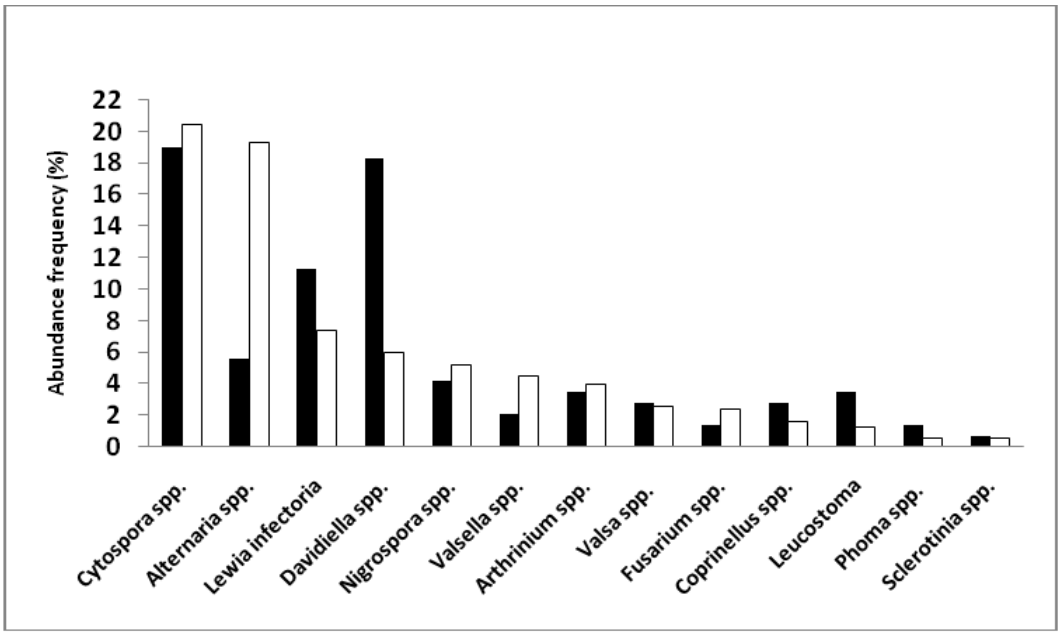


Figure 3-3. Fungal abundance in willow stem/bark and leaf samples as determined by culture-dependent/PCR amplification (□) and DGGE (■) methods
(DGGE analysis is based on percentage of all bands detected from Alberta and SK2 sampling sites).

The migration pattern of fungal isolates obtained from environmental samples was almost similar to pure cultures for the same sampling sites. Most of the stem/leaf samples had multiple bands (1 to 7) on DGGE gels. Samples that originated from the University of Saskatchewan multi-clonal willow plantation, particularly clones *India*, *SV1* and *Juliet*, yielded multiple bands including several dominant bands and one or two faint bands. The majority of fungal isolates responded to the ITS rDNA primer set. The obtained migration patterns of DNA amplicons using the LS1-GC/LR5 primer pair directly discriminated *Coniocheta* and *Protoventuria* species whereas *Valsa* and *Valsella* species were more distinguishable using NS rDNA primer pair. In contrast with other fungal species, the DGGE profile of *Cytospora chrysosperma* and *Leucostoma niveum* had bands in lower areas (GC-rich region).

Both species richness (S) and Shannon diversity indexes (H´) in diseased samples were higher than in healthy plants. In addition, these indexes were higher in diseased stems followed by diseased leaf samples compared to healthy ones. The greatest fungal biodiversity (Shannon index) was found in Alberta among diseased leaves (1.12) followed by clone *India* in healthy leaves (1.09) and then in Alberta and clone *Hotel* (both 1.08) in diseased stems. The highest species richness was calculated for Alberta (16.02) among diseased stems whereas the clones *Hotel* and *SX64* had the lowest value (Table 3-5, A and B).

Table 3-5. Shannon's diversity (H') and Species richness (S) indexes for fungi found on willow healthy vs. diseased stem/bark (A) and leaf (B) samples (2007 sampling)

A	**Healthy Leaf**				**Diseased Leaf**			
Plantation	**Number of isolates**	**Number of Taxa**	**Shannon diversity**	**Species richness**	**Number of isolates**	**Number of Taxa**	**Shannon diversity**	**Species richness**
Ab	11	5	0.6	4.30	11	8	1.12	8.04
Sk1	6	6	0.78	6.41	7	4	0.56	3.53
Mb	16	7	0.9	5.00	11	5	0.71	3.85
Charlie	9	5	0.62	4.21	24	13	1.05	8.70
Hotel	1	1	Uncomputable	0.00	8	6	0.75	5.56
India	22	13	1.09	8.96	22	9	0.92	5.97
SV1	11	9	0.93	7.96	22	9	0.88	5.97
Juliet	12	6	0.79	4.63	19	12	1.06	8.59
SX64	1	1	Uncomputable	0.00	15	6	0.69	4.24
SX61	8	6	0.75	5.56	8	4	0.39	3.33
Total	97	59	6.46	47.03	147	76	8.13	57.78
B	**Healthy Stem**				**Diseased Stem**			
Plantation	**Number of isolates**	**Number of Taxa**	**Shannon diversity**	**Species richness**	**Number of isolates**	**Number of Taxa**	**Shannon diversity**	**Species richness**
Ab	13	7	1.04	6.19	32	21	1.08	16.02
Sk1	0	0	Uncomputable	0.00	3	3	0.48	4.17
Mb	4	2	0.3	1.67	16	8	0.69	5.83
Charlie	13	8	0.58	6.31	27	9	0.81	5.59
Hotel	2	2	0.3	3.33	27	10	1.08	6.29
India	15	12	1.04	9.32	21	12	1.01	6.33
SV1	18	10	0.95	7.14	21	11	0.97	7.58
Juliet	6	5	0.68	5.13	25	11	0.99	7.14
SX64	3	3	0.48	4.17	24	11	0.96	7.25
SX61	3	3	0.48	4.17	38	13	1.06	7.59
Total	77	52	5.85	47.43	234	109	9.13	73.79

3.5.2.1. Composition of Fungal Communities in Relation to Different Locations and Clones

The cultural method indicated that Alberta had the highest diversity compared to the other locations. The majority of identified fungal isolates belong to *Pleosporales* and *Diaporthales* troughout sampling sites. *Diaporthales* isolates were recorded from all clones except *Juliet* and *SV1*, whereas *Pleosporales* were the most frequent fungal isolates (Figure 3-4). *Capnodiales* occured in all clones except *Juliet* while *Mucorales and Coniochaetales* were exclusively found in *Juliet* (Figure 3-5, C). *Hypocreales* was only present in Alberta and SK2 (Figure 3-5, B). In Alberta, Manitoba, and SK1, the majority of isolated species belonged to *Alternaria* spp. However, *Cytospora* spp. was identified as the most frequent species in SK2. .

DGGE results showed the highest fungal diversity at the SK2 location. *Hypocreales* was associated with diseased stem and leaf samples from *Juliet*, *India*, and *SV1*. *Cytospora* spp. was the major fungal taxon identified in diseased samples in SK2.

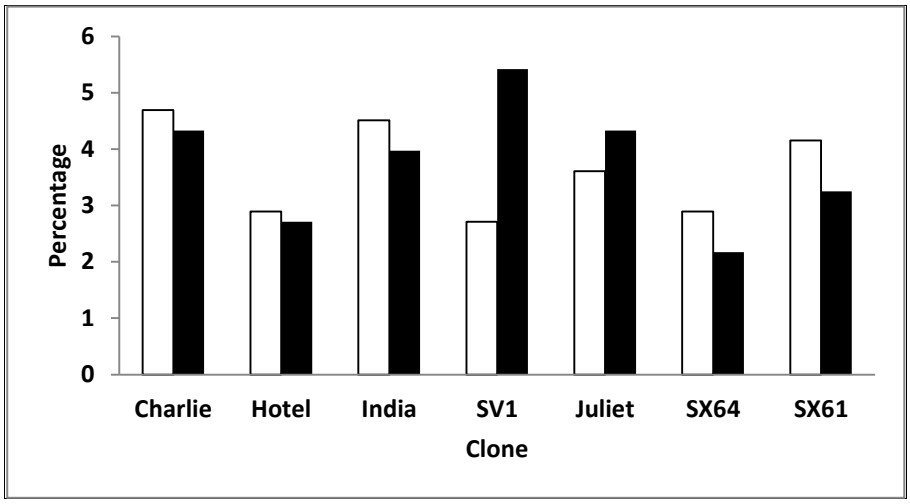
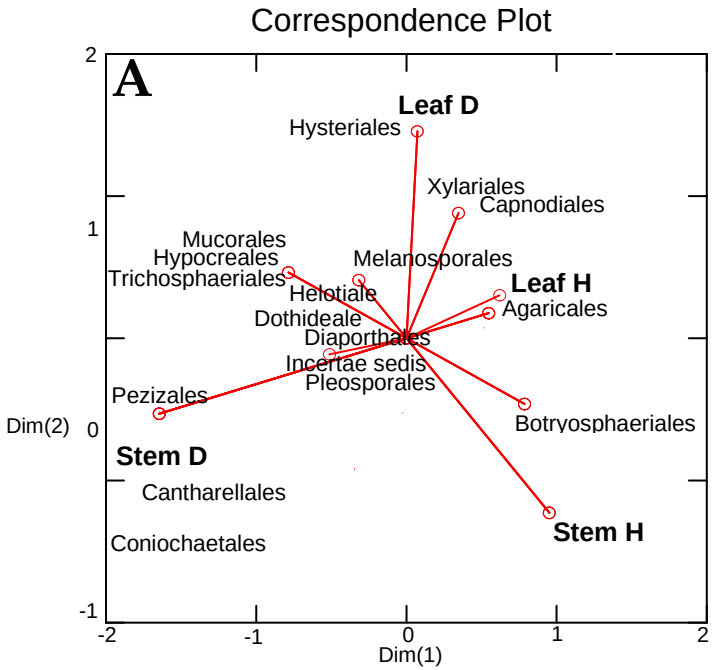
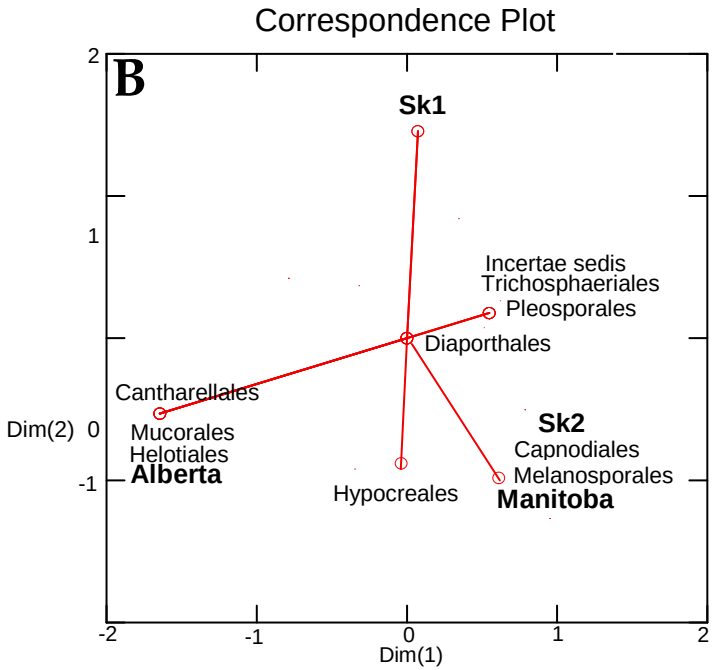


Figure 3-4. The most frequent ascomycetous *Diaportales* (□) and *Pleosporales* (■) taxa on stem/bark and leaf of different willow clones under SRIC.





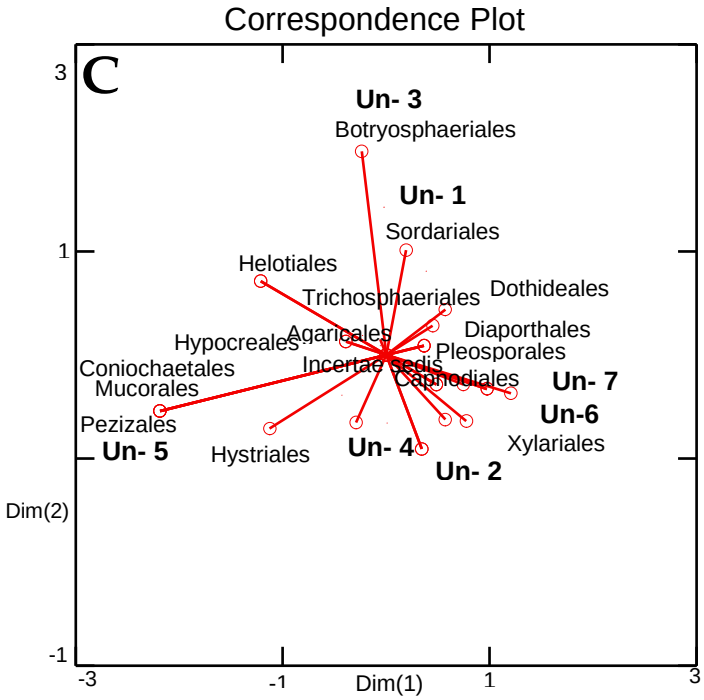


Figure 3-5. Correspondence analysis (CA) of fungal communities associated with aerial tissues of willow grown under SRIC.

(**A**) CA of the relationship between isolated fungi associated with plant health status (healthy and diseased), (**B**) CA of the relationship between the isolated fungi and province (plantation location) including; Saskatchewan (Sk1), Manitoba (Mb), Alberta (Ab), and clone *Hotel* (Sk2) in the University of Saskatchewan plantation, and (**C**) The University of Saskatchewan willow plantation seven clones including; 1- *Charlie* 2- *Hotel* 3- *India* 4- *SV1* 5- *Juliet* 6- *SX64* and 7- *SX61*, 2007 sampling.

3.5.2.2. Composition of Fungal Communities in Relation to Willow Clones Health Status

The isolation of cultivable fungi and DGGE fingerprinting from stem/leaf samples showed that fungal communities from diseased plants tended to be more diverse than those from healthy plants (Figure 3-5, A). Accordingly, fungal community structure was directly related to the health status of plants. The most frequent fungal inhabitants in both healthy and diseased stem/leaf belonged to *Pleosporales* and *Diaporthales*. *Cytospora* spp. and *Alternaria* spp. were recorded as the most frequently isolated species in diseased and healthy stem/leaf samples respectively.

Valsa spp., *Diaporthe fibrosa, Glomerella acutata, G. cingulata,* and *Botrytis byssoidea* were isolated only from diseased stems whereas *Marssonia populi, Glonium pusillum, Pithya cupressina,* and *Trichoderma longibrachiatum* were separated only from diseased leaves. The total number of potentially pathogenic isolated fungi from stem/leaf samples accounted for 32.5% according to the Hubes checklist (1983) (Table 3-6). Fungal endophytes were calculated to comprise 2.5% of all fungi among healthy stems and leaves. Fungal species including *Pseudodiplodia* sp., *Humicola fuscoatra,* and *Chaetomium globosum* were identified only in healthy stems. *Cladosporium, Epicoccum nigrum, Davidiella macrospora, Aureobasidium pullulans,* and *Fimetariella rabenhorstii,* were only found in healthy leaves (Tables 3-7 and 3-8).

A small number of fungal isolates belonged to *Basidiomycota* (*Ceratobasidium* sp. and *Coprinellus* spp.).

Table 3-6. Potentially pathogenic fungi (Hubes's species checklist, 1983) isolated from diseased willow stem/bark and leaf samples

Order	Family	Species
Diaporthales	*Valsaceae*	*Leucostoma persoonii, L. niveum, Valsa malicola, V. salicina, V. sordida, V.ambiens, Leucostoma persoonii, L. niveum, Cytospora pruinosa, C. translucens, C. chrysosperma, C.eutypelloides, and Valsella melostoma.*
Incertae sedis	*Glomerellaceae*	*Glomerella cingulata* and *G. acutata*
Hypocreales	*Nectriaceae*	*Nectria haematococca*
Pleosporales	*Incertae sedis*	*Phoma medicaginis* and *Phoma glomerata*
Helotiales	*Dermateaceae*	*Marssonia populi*

Table 3-7. Fungal diversity in willow healthy vs. diseased aerial (stem/bark & leaf) tissues based on OTUs-phenotypic characteristics and rDNA sequences (2007 and 2008)

Description	Total number of cultures isolated	OUT	SMCD	DNA sequence	Associated accession number	Percent identity
Phylum Ascomycota						
Botryosphaeriales, Botryosphaeriaceae						
Microdiplodia	3	1.9	2573	ITS	FJ228194.1	98%
Capnodiales, Davidiellaceae						
Cladosporium malorum strain STE-U 4571	2	M13	2523	LS	AY251081.2	97%
Cladosporium variabile	2	M5	2524	ITS	EF679403.1	85%
Davidiella macrospora	33	2.1	2540	ITS	EU167591.1	99%
Coniochaetales, Coniochaetaceae						
Coniochaeta punctulata	1	23.4	2525	ITS	AJ875231.1	97%
Diaporthales, Valsaceae						
Cytospora chrysosperma isolate dx-22	2	31.2	2532	ITS	FJ441005.1	93%
Cytospora eutypelloides strain IMI140798	1	25.1	2536	ITS	DQ243806.1	82%
Cytospora chrysosperma	33	29.1	2531	ITS	FJ478104.1	97%
Cytospora chrysosperma xsd08013 strain 1	26	15.1	2533	ITS	EU918709.1	96%
Cytospora chrysosperma xsd08013 strain 2	2	32.3	2534	ITS	FJ478104.1	99%
Cytospora chrysosperma xsd08013 strain 3	12	21.1	2535	ITS	FJ478104.1	87%
Cytospora pruinosa	1	13.5	2537	ITS	EU552121.1	93%
Cytospora translucens strain 1	9	27.1	2538	ITS	EF447404.1	90%
Cytospora translucens strain 2	27	32.1	2539	ITS	EF447403.1	98%
Diaporthe fibrosa	1	M10	2541	LS	AF408351.1	99%
Leucostoma niveum strain 1	1	25.6	2565	LS	AF362558.1	99%
Leucostoma niveum strain 2	2	13.1	2566	ITS	DQ243794.1	98%
Leucostoma persoonii strain 1	1	12.4	2567	ITS	EF447375.1	96%
Leucostoma persoonii strain 2	3	18.5	2568	ITS	AM236582.1	83%
Valsa ambiens	1	17.2	2598	NS	DQ862056.1	98%
Valsa malicola	2	15.4	2599	ITS	EF447416.1	88%
Valsa malicola isolate 256	1	30.5	2600	ITS	EF447416.1	97%

Valsa salicina strain 1	1	15.10	2601	ITS	EF447364.1	99%
Valsa salicina strain 2	2	25.5	2602	ITS	EF447417.1	99%
Valsa sordida	7	22.1	2603	ITS	EF447418.1	81%
Valsella melostoma	1	32.6	2604	ITS	AF191184.1	98%
Valsella salicis isolate AFTOL-ID 2132	24	18.2	2605	NS	DQ862057.1	93%
Dothideales, Dothioraceae						
Aureobasidium pullulans strain 1	5	16.2	2518	ITS	FJ228168.1	95%
Aureobasidium pullulans strain 2	1	25.4	2519	LS	FM212450.1	90%
Helotiales, Dermateaceae						
Marssonia populi	1	11.2	2572	ITS	EU732730.1	99%
Helotiales, Sclerotiniaceae						
Botrytis byssoidea	5	34.1	2520	ITS	FJ169671.1	99%
Monilinia laxa	1	M20	2574	ITS	EF153017.1	94%
Sclerotinia sclerotiorum	2	M24	2593	LS	AF431951.1	94%
Sclerotinia sp.	1	12.7	2594	ITS	AJ279480.1	99%
Hypocreales, Hypoceraceae						
Trichoderma longibrachiatum	1	19.1	2597	ITS	FJ459970.1	93%
Hypocreales, Nectriaceae						
Fusarium equiseti	10	12.1	2555	ITS	EU326202.1	98%
Fusarium sp. 19010	2	12.3	2556	ITS	EU750695.1	99%
Fusarium tricinctum	1	12.5	2557	ITS	DQ093675.1	100%
Gibberella avenacea	2	9.3	2558	ITS	EU255802.1	98%
Nectria haematococca strain 1	4	4.2	2576	ITS	FJ441642.1	99%
Nectria haematococca strain 2	4	11.1	2577	ITS	AF130142.1	98%
Hysteriales, Hysteriaceae						
Glonium pusillum	2	4.8	2561	ITS	EU552134.1	99%
Incertae sedis, Apiosporaceae						
Apiospora montagnei	4	M4	2511	ITS	FJ228174.1	91%
Arthrinium phaeospermum strain 1	2	5.5	2512	ITS	AJ279447.1	97%
Arthrinium phaeospermum strain 2	4	21.2	2513	LS	AY083832.1	95%
Arthrinium sacchari	5	7.1	2514	ITS	EF076712.1	99%
Arthrinium sacchari strain FBC.143	5	9.5	2515	ITS	EF076710.1	100%
Arthrinium sp. strain 1	1	4.5	2516	ITS	AB220267.1	84%
Arthrinium sp. strain 2	5	5.2	2517	ITS	AF455478.1	99%
Incertae sedis, Glomerellaceae						

Glomerella acutata	4	M21	2559	ITS	AM991136.1	93%
Glomerella cingulata	6	M14	2560	ITS	AJ301952.1	99%
Incertae sedis, Incertae sedis						
Pseudodiplodia sp.	1	24.1	2590	ITS	EU754201.1	85%
Melanosporales, Ceratostomataceae						
Persiciospora africana strain ATCC64691	2	M2	2583	LS	AY015631.1	98%
Pezizales, Sarcoscyphaceae						
Pithya cupressina	1	15.12	2589	ITS	U66009.1	91%
Pleosporales, Incertae sedis						
Leptosphaerulina trifolii	3	9.1	2564	ITS	AY131203.1	95%
Phoma glomerata	1	15.11	2587	ITS	AY183371.1	99%
Phoma medicaginis	2	10.3	2588	ITS	EU167575.1	95%
Pleosporales, Leptosphaeriaceae						
Coniothyrium diplodiella isolate T25	6	25.2	2527	ITS	FJ462758.1	99%
Pleosporales, Montagnulaceae						
Paraphaeosphaeria michotii	5	19.2	2582	ITS	AF250829.1	90%
Pleosporales, Phaeosphaeriaceae						
Phaeosphaeria avenaria	22	4.1	2584	ITS	U77359.1	99%
Phaeosphaeria pontiformis	1	28.5	2585	ITS	AJ496632.1	87%
Phaeosphaeria sp.	2	M8	2586	ITS	EF432300.1	99%
Stagonospora sp.	1	17.1	2595	ITS	AJ496626.1	99%
Pleosporales, Pleosporaceae						
Alternaria brassicae	1	16.5	2500	ITS	AY154714.1	99%
Alternaria sp. strain 1	1	28.6	2501	ITS	DQ491089.1	99%
Alternaria sp. strain 2	69	1.1	2502	ITS	EF432293.1	99%
Alternaria sp. strain 3	4	26.3	2503	ITS	EF432287.1	95%
Alternaria sp. strain 4	1	28.3	2504	ITS	FJ037742.1	96%
Alternaria sp. strain 5	2	M1	2505	ITS	FJ196613.1	94%
Alternaria sp. strain 6	2	M3	2506	ITS	FJ467349.1	94%
Alternaria sp. strain 7	2	M25	2507	ITS	EF432288.1	94%
Alternaria tenuissima	5	M19	2508	ITS	AY154711.1	99%
Alternaria triticina	20	1.4	2509	ITS	AY154695.1	99%
Epicoccum nigrum strain 1	5	4.3	2542	ITS	EU232716.2	99%
Epicoccum nigrum strain 2	1	4.4	2543	ITS	AF455403.1	99%
Epicoccum nigrum strain 3	1	M12	2544	ITS	AJ279448.1	98%
Epicoccum nigrum strain 4	2	M16	2545	LS	FJ424261.1	99%

Epicoccum nigrum strain 5	1	M17	2546	LS	FM991735.1	97%
Epicoccum nigrum isolate 2691	1	9.4	2547	ITS	EU272495.1	99%
Epicoccum sp. 6/97-74	2	10.4	2548	ITS	AJ279463.1	98%
Lewia infectoria strain 1	34	1.3	2569	ITS	AY154718.1	99%
Lewia infectoria strain 2	4	4.9	2570	ITS	AY154691.1	99%
Lewia infectoria strain 3	3	28.2	2571	ITS	AY154691.1	99%
Pyrenophora avenae	3	10.1	2591	ITS	EF452453.1	99%
Pyrenophora teres	1	10.7	2592	ITS	EF452474.1	99%
Stemphylium solani	2	10.8	2596	ITS	AF203450.1	97%
Sordariales, Chaetomiaceae						
Chaetomium globosum	1	15.17	2522	ITS	FJ228182.1	98%
Humicola fuscoatra	1	15.13	2562	ITS	EF120414.1	91%
Sordariales, Lasiosphaeriaceae						
Fimetariella rabenhorstii	1	13.4	2549	ITS	EU781677.1	88%
Trichosphaeriales, Incertae sedis						
Nigrospora oryzae strain 1	11	6.1	2578	ITS	DQ219433.1	100%
Nigrospora oryzae strain 2	14	8.1	2579	ITS	EU272488.1	99%
Nigrospora oryzae isolate AFTOL-ID 2179	2	M23	2580	LS	FJ176892.1	96%
Nigrospora oryzae strain CBS	2	M22	2581	ITS	DQ219433.1	96%
Xylariales, Xylariaceae						
Anthostomella conorum	1	13.2	2510	ITS	EU552099.1	94%
Coniolariella hispanica	1	3.1	2526	ITS	FJ172294.1	96%
Hypoxylon fuscum	1	7.2	2563	ITS	AF201715.1	97%
Phylum Basidiomycota						
Agaricales, Agaricaceae						
Coprinellus sp.1	2	24.2	2530	LS	AJ406565.1	99%
Coprinellus sp.2	4	5.1	2529	ITS	EU436684.1	99%
Coprinellus curtus	3	4.7	2528	ITS	AB266447.1	96%
Cantharellales, Ceratobasidiaceae						
Ceratobasidium sp.	1	31.6	2521	ITS	DQ093646.1	98%
Phylum Zygomycota						
Mucorales, Mucoraceae						
Mucor fragilis	2	31.3	2575	ITS	AJ608958.1	78%
Endophytes						
Fungal endophyte sp. D2-1B1-10-1	4	22.2	2550	ITS	FJ025339.1	83%
Fungal endophyte sp. D5-1B1-3-1	1	19.9	2551	ITS	FJ025282.1	99%
Fungal endophyte sp. M13-3311-A	7	26.1	2552	ITS	FJ025362.1	95%

Fungal endophyte sp. M13-3311-B	1	29.5	2553	ITS	FJ025362.1	81%
Fungal endophyte sp. O26-3333	1	31.7	2554	ITS	FJ025263.1	93%

● OUT (Operational Taxonomic Unit) numbers from 1.1 to 34.1 (Sk and AB) and M1 to M25 (Manitoba).

Pure cultures were grouped into operational taxonomic units based on their morphology and with the aid of microscopy for doubtful cultures (Vujanovic et al., 2007).

Table 3-8. Fungal taxa diversity on stem/bark and leaf using PCR-DGGE method

Description	Total number of cultures isolated	OTU	DNA sequence	Associated accession number	Percent identity
Phylum Ascomycota					
BotryosphaerialesBotryosphaeriaceae					
Microdiplodia sp. (endophyte)	1	U5.1	LS	FJ228194.1	98%
Capnodiales, Davidiellaceae					
Cladosporium cladosporioides isolate ClaE	3	U15.6	NS	FJ717696.1	98%
Cladosporium cladosporioides strain Y1-14	2	U27.6	ITS	GU723437.1	97%
Cladosporium cucumerinum	2	U6.6	ITS	GU594747.1	95%
Cladosporium cucumerinum strain 871915	2	U13.2	ITS	GU594747.1	99%
Cladosporium sp. 6027	1	U15.4	NS	FJ235525.1	98%
Cladosporium sp. strain 1	1	U15.8	NS	GU322367.1	97%
Cladosporium sp. strain 2	2	A4.2	ITS	GU212394.1	100%
Cladosporium sphaerospermum	1	U15.7	NS	AY251098.2	98%
Davidiella macrospora strain 1	4	U1.1	ITS	FR667968.1	96%
Davidiella macrospora strain 2	4	U24.1	ITS	EU167591.1	99%
Davidiella macrospora strain 3	13	U13.1	ITS	FR667968.1	99%
Davidiella tassiana strain 1	1	U6.5	ITS	GU566258.1	99%
Davidiella tassiana strain 2	2	U15.1	NS	EU343080.1	98%
Davidiella tassiana strain 3	1	U15.9	NS	EU343092.1	100%
Davidiella tassiana strain 4	1	U25.1	LS	EU343661.1	83%
Diaporthales, Valsaceae					
Cytospora chrysosperma strain 1	7	U4.1	ITS	EF447416.1	97%
Cytospora chrysosperma strain 2	11	U13.4	LS	FJ478104.1	97%
Cytospora chrysosperma strain 3	8	U20.1	LS	EF447416.1	98%
Cytospora chrysosperma strain 4	1	U23.2	ITS	EF447416.1	88%
Leucostoma persoonii	5	U6.3	ITS	EF447375.1	96%
Valsa malicola isolate 256	1	A8.3	ITS	EF447416.1	97%
Valsa salicina	1	U10.2	LS	EF447364.1	99%
Valsa sordida	1	A4.4	ITS	FJ755274.1	92%
Valsa sordida strain CZ421	1	U8.1	LS	FJ755274.1	99%

Valsella melostoma	1	U4.2	ITS	AF191184.1	98%
Valsella salicis strain AR 3514	1	U8.3	LS	EU255210.1	99%
Valsella salicis isolate AFTOL-ID 2132	1	U22.1	ITS	DQ862057.1	93%
Dothideales, Dothioraceae					
Aureobasidium pullulans strain 1	1	A1.1	ITS	EF690466.1	97%
Aureobasidium pullulans strain 2	1	A9.1	ITS	FM212450.1	90%
Eurotiales, Trichocomaceae					
Aspergillus niger isolate 6	1	U25.2	LS	HM347449.1	92%
Helotiales, Dermateaceae					
Marssonia populi	1	U27.1	ITS	EU732730.1	99%
Helotiales, Sclerotiniaceae					
Sclerotinia sp.	1	U8.6	LS	AJ279480.1	99%
Hypocreales, Nectriaceae					
Fusarium equiseti	1	U27.3	ITS	EU326202.1	98%
Fusarium sp. 19010	1	U19.3	LS	EU750695.1	99%
Nectria haematococca strain 1	3	U8.2	ITS	AF130142.1	99%
Nectria haematococca strain 2	3	A6.3	ITS	FJ441642.1	99%
Incertae sedis, Apiosporaceae					
Arthrinium sp.	4	U5.2	LS	AF455478.1	99%
Arthrinium sacchari	1	U19.1	LS	EF076710.1	99%
Pleosporales, Incertae sedis					
Phoma glomerata	1	U13.3	ITS	AY183371.1	99%
Phoma medicaginis	1	U27.2	ITS	EU167575.1	95%
Pleosporales, Montagnulaceae					
Paraphaeosphaeria michotii	1	U2.2	ITS	AF250829.1	90%
Pleosporales, Phaeosphaeriaceae					
Phaeosphaeria avenaria	1	U15.2	NS	EF432300.1	99%
Pleosporales, Pleosporaceae					
Alternaria alternata strain PP135b	1	U20.3	LS	FJ890364.1	97%
Alternaria maritima strain CBS 126.60	1	U20.5	LS	GU456317.1	99%
Alternaria sp.	6	U6.1	ITS	GQ865634.1	99%
Epicoccum nigrum	3	A13.2	LS	AF455403.1	99%
Epicoccum sp. 6/97-74	1	U16.3	ITS	AJ279463.1	98%
Lewia infectoria strain 1	6	U12.1	ITS	AY154691.1	99%
Lewia infectoria strain 2	8	A13.1	ITS	AY154718.1	99%
Lewia infectoria strain 3	2	A8.1	LS	AY154691.1	99%

Trichosphaeriales, Incertae sedis					
Nigrospora oryzae	6	U17.1	ITS	DQ219433.1	100%
Phylum Basidiomycota					
agaricales, Psathyrellaceae					
Coprinellus curtus	1	U16.1	ITS	AB266447.1	96%
Coprinellus sp.	4	U6.2	ITS	EU436684.1	99%

● OUT: Operational Taxonomic Unit

3.5.2.3. Taxonomical Groups of Fungi Associated with Willow Stem/Bark and Leaf

Phylogenetic trees were generated with sequences obtained from the amplification of the ITS rDNA region. One of the four most parsimonious trees with a set of 10 sequences belonged to *Incerta sedis* (Figure 3-6). The *Diaporthales* distance tree consists of the 19-sequences belonging to the stem/bark pathogenic taxa. The majority of them belong to the family *Valsaceae* with 18 fungal species, which are phylogeneticaly and functionally closely related *Diapothaceae* family represented with only one fungal species (*Diaporthe fibrosa)* (Figure 3-7). *Pleosporales* diverges within the clade to the families of *Pleosporaceae, Incertae sedis, Montagnulaceae, Leptosphaeriaceae,* and *Phaeosphaeriaceae* (Figure 3-8). *Basidiomycota* consisted of four fungal taxa including and *Ceratobasidium* sp. and *Coprinellus* spp. (Figure 3-9).

Unweighted arithmetic average clustering (UPGMA) demonstrated relationships in the occurrences of isolated fungal taxa in both healthy and diseased samples using culture-based method. The dendrogram demonstrated the distinction between different plantations based on the presence or absence of fungal taxa. The genetic similarities ranged from 78% to 100% and it revealed 8 clusters. The most distinct cluster was formed by the fungal species from Manitoba; this cluster was mostly monophylic and completely separate from the rest of the group. The fungal variations in Saskatoon (SK1) and Alberta plantations were highly inter-related and almost similar. According to the obtained dendrogram, clusters from Saskatoon, Alberta, and two university clones including *Hotel* (SK2) and *SX64* comprised one group (Figure 3-10).

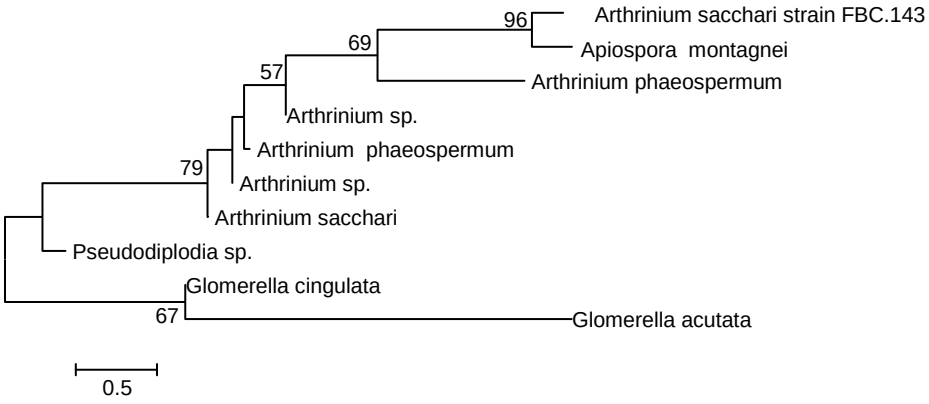


Figure 3-6. Bootstrap tree (maximum parsimony analysis) showing the relationships within *Incertae sedis* fungal taxa (Families: *Apiosporaceae, Incertae sedis*, and *Glomerellaceae*) found in stem/bark and leaf samples. The tree constructed on the basis of the fungal internal transcribed spacer rDNA sequences. Bootstrap values were obtained from 500 replications. The numbers above the clades are the bootstrap values. See table 3-7 for the description of the fungal isolates. Distance tree for

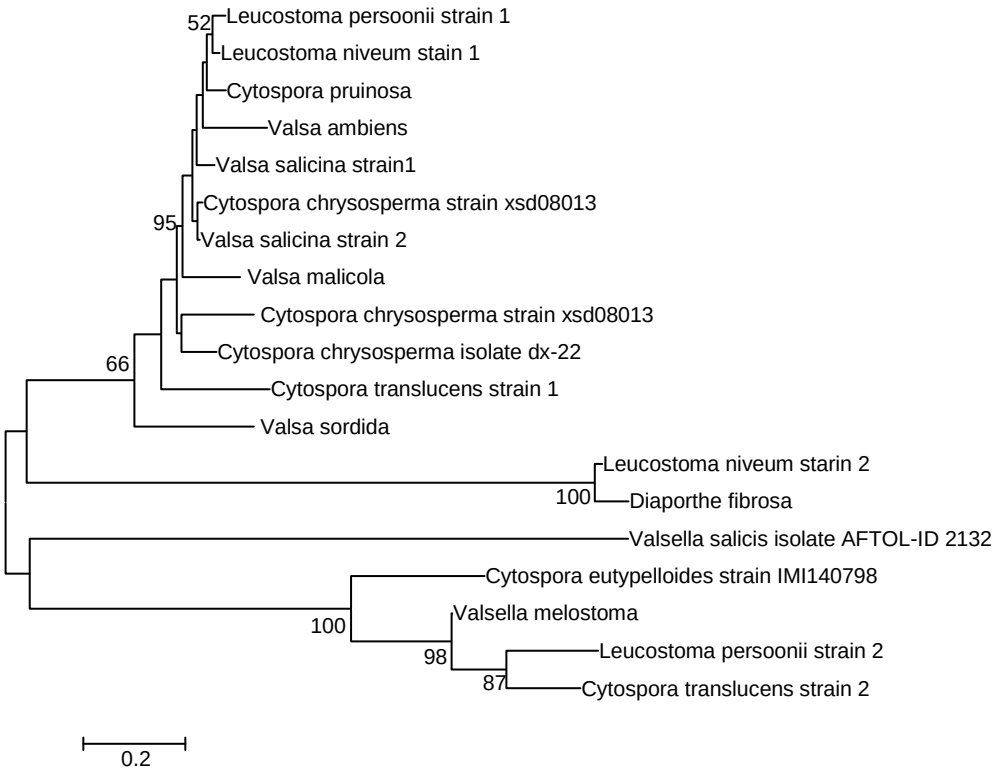


Figure 3-7. Bootstrap tree (maximum parsimony analysis) showing the relationships within 19 *Diaporthales* fungal taxa (Families: *Valsaceae* and *Diaporthaceae*) found in stem/bark and leaf samples. The tree constructed on the basis of the fungal internal transcribed spacer rDNA sequences. Bootstrap values were obtained from 500 replications. The numbers above the clades are the bootstrap values. See table 3-7 for the description of the fungal isolates.

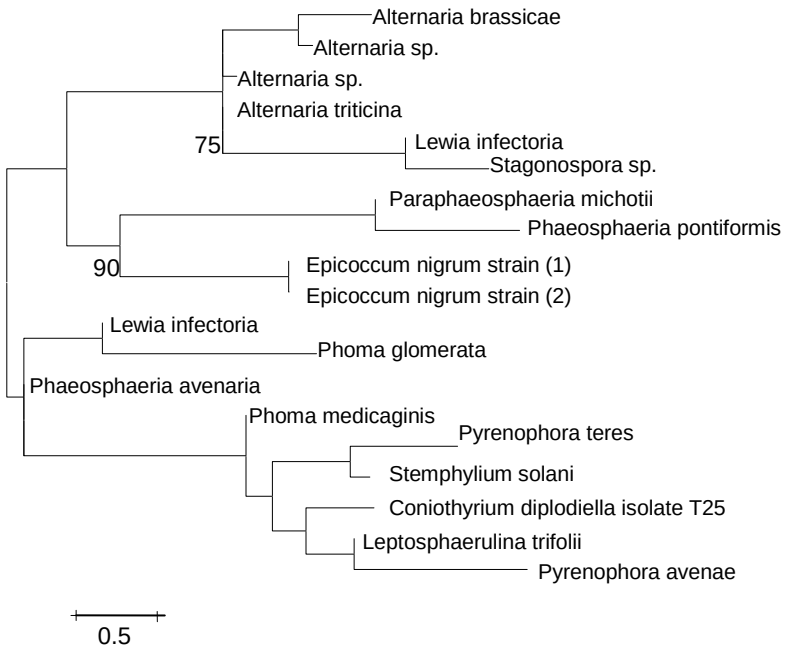


Figure 3-8. Bootstrap tree (maximum parsimony analysis) showing the relationships within *Pleosporales* fungal taxa (Families: *Pleosporaceae, Incertae sedis, Montagnulaceae, Leptosphaeriaceae,* and *Phaeosphaeriaceae*) found in stem/bark and leaf samples. The tree constructed on the basis of the fungal internal transcribed spacer rDNA sequences. Bootstrap values were obtained from 500 replications. The numbers above the clades are the bootstrap values. See table 3-7 for the description of the fungal isolates.

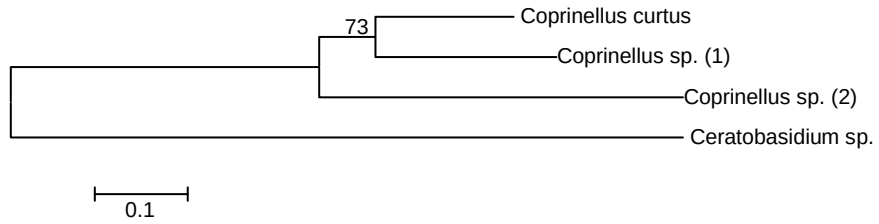


Figure 3-9. Bootstrap tree (maximum parsimony analysis) showing the relationships within *Basidiomycota* fungal taxa found in stem/bark and leaf samples. The tree constructed on the basis of the fungal internal transcribed spacer rDNA sequences. Bootstrap values were obtained from 500 replications. The numbers above the clades are the bootstrap values. See table 3-7 for the description of the fungal isolates.

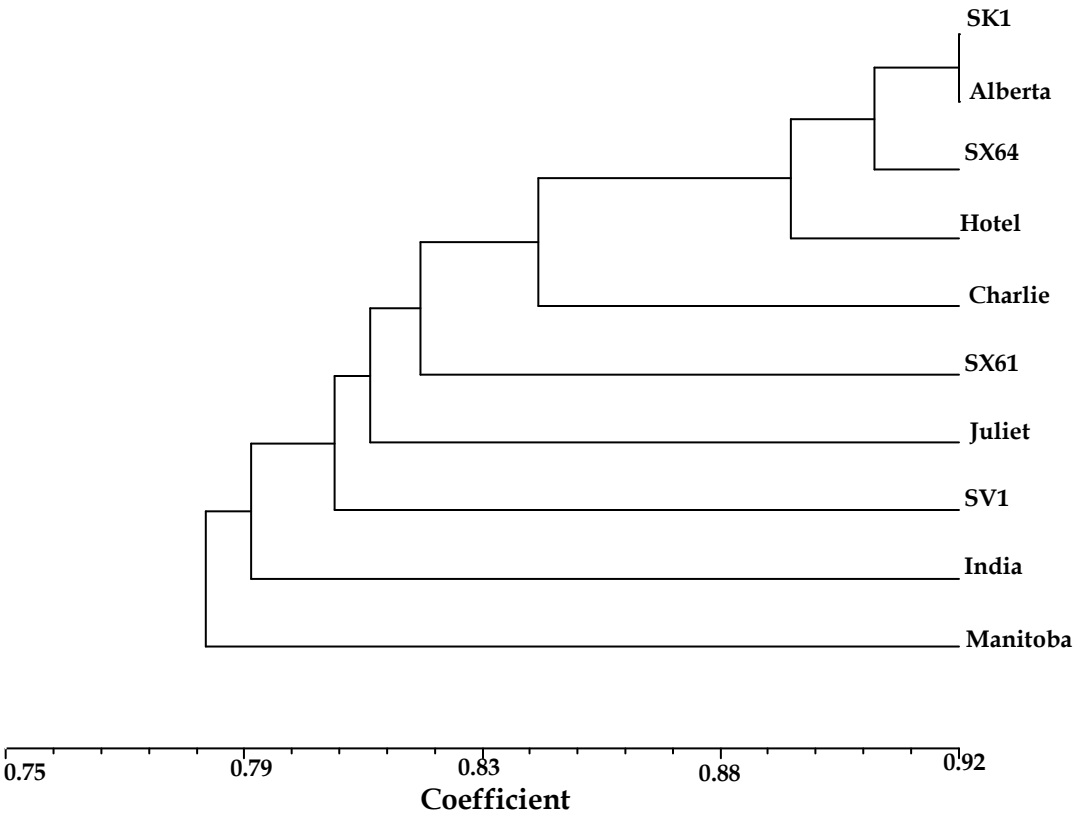


Figure 3-10. Unweighted arithmethic average clustering **(**UPGMA) of fungal species isolated from healthy and diseased above-ground tissues of willow clones at three provincial SRIC- locations (SK, AB, MB). The distance used is the Jaccard's coefficient of similarity. Cluster dendrogram was based on presence/absence of fungal communities. Short linkage distance means greater degree of similarity.

3.6. Discussion

3.6.1. Willow Cuttings

There are a limited number of studies about harmful fungal effects on willow cuttings. This study pioneers research into determining the mycodiversity on willow cuttings. In this study, fast-growing *Ascomycota* taxa were definitely the most abundant and diverse fungi among our findings related to willow cuttings with different isolated fungal taxa. Similar fungal taxa were previously reported as known pathogenic agents in willow biomass plantations in eastern Canada (Vujanovic and Labrecque, 2008).

In this study, some potentially pathogenic fungi were identified from willow cuttings, such as *Phoma glomerata* and *P. cava* which was reported to be a weak parasite of willow trees that usually occur after infection of *Fusicladium* spp. (teleomorph*: Venturia*) or other mitosporic pathogens including *Cladosporium* spp. (Hubes, 1983; Vujanovic and Labrecque, 2008). However, *Fusicladium* was not identified in willow cuttings in our study, whereas *Cladosporium* was abundant. Also, three different species from the *Glomerellaceae* family (*Glomerella acutata, G.cingulata,* and *Colletotrichum circinans*) were found in cutting samples. *G.cingulata* (anamorph: *Colletotrichum*) causes stem black canker and leaf spots (anthracnose) usually in the presence of *Fusicladium* spp. (Vujanovic and Labrecque 2008). *Glomerella cingulata* was previously isolated from *Salix alba*, *S. babylonica*, and *S. fragilis* in Nova Scotia, Quebec, and British Columbia (Hubes, 1983) and reported as a pathogenic fungus on willow tissues (stems and leaves) in Canada and USA (Vujanovic and Labrecque, 2002; Farr et al., 1989).

From cutting samples, we also separated *Nectria vilior*, *Neonectria ramulariae,* and *Bionectria ochroleuca,* whose pathogenicity has not been investigated in willow yet but *Nectria* spp. such as *N.cinnabarina, N.coccinea, and N.galligena* are associated with the presence of perennial cankers with greatly swollen irregular callus edges (Hubes, 1983). Besides, *Fusarium* spp. (teleomorph: *Gibberella* sp.) such as *F. larvarum* and *G. avenacea* (anamorph: *Fusarium avenaceum*) have been isolated from willow cuttings. *F. lateritium* has been reported as the causative agent of stem and branch cankers on willows in both western Canada (Funk, 1981) and eastern Canada (Vujanovic and Labrecque 2008). In general, *Fusarium* spp. are not considered as primary parasites on willow, but they cooperate with other aggressive primary pathogens that weaken the host (Hubes, 1983). Furthermore, we isolated several *Coniochaeta* (anamorph: *Lecythophora*) species which are known to be pathogens on woody hosts. This group of fungi

can be considered as a threat for biomass (stem/wood) production in SRIC willow plantations. It has never been reported in association with willow. Also, these species can frequently occur in some agricultural crops such as peach and nectarine trees (Damm et al., 2010) which may be important for the possible distribution of this pathogen from agricultural crops to willow trees or vice versa.

Among the fast-growing *Ascomycota* fungi which were abundantly isolated in this study, some of them are not reported as common pathogenic fungi in willow tissues, including cuttings. However some of these taxa such as *Fusarium* spp. (*Gibberella*), *Nectria* spp. and *Lecythophora* spp. may act as opportunists and may use cuttings as a temporary host. Introducing new fungal diseases in willow plantations may also occur, due to the lack of previous exposure and lack of defense against the opportunistic fungi. The risk of this type of interaction may require serious attention to importation processes, to decrease the chance of establishment of non-indigenous fungi in SRIC willow plantations.

We also found *Phialemonium dimorphosporum* in willow cuttings. This fungal species is close to *Phialophora* which causes cutaneous mycosis in humans but also cystic ulcerative mycosis (Shin et al., 2005). From a human safety standpoint, the mentioned fungus is important for cutting inspection before introduction to the plantation. It must be pointed out that it has health significance for people who manipulate cuttings such as farm workers. Prevention of those health problems may be possible with good management using molecular methods.

In the present study, various fungal taxa were successfully recovered from willow cutting using the PCR-DGGE method. According to the results, willow cuttings were recognized as a natural habitat for diverse fungi such as *Hypocreales, Coniochaetales, Dothideales, Capnodiales, Incertae sedis, Helotiales,* as well as *Agaricales* which indicated the probable interaction between potentially beneficial and pathogenic fungi in willow trees. Our results indicated that *Lecythophora* spp. was the most prevalent fungal species found in willow cuttings using the DGGE method whereas *Kabatiella microsticta* was abundantly found using the culture-based method. We could not find any report regarding isolation and pathogenicity of these fungi in willow plants. However, *L.luteoviridis* was previously isolated from kiwifruit plants and reported as being responsible for wood discoloration in *Picea abies* (Prodi et al., 2008).

Our results support the theory that major pathogenic fungal isolates may originate from willow cuttings. As a consequence, willow cuttings may play an important role as a carrier for

distribution of fungal pathogens. Introducing new fungal agents to agricultural fields is another important aspect regarding colonization of fungal pathogens in willow cuttings. On the other hand, some isolated fungi such as *Penicillium* sp., *Penicillium lanosum, Penicillium commune, Coprinellus* sp., *Cladosporium cladosporioides* and several fungal endophytes are carried by willow cuttings as potentially beneficial fungi.

Results showed that there were some differences and similarities among fungal community structures using culture-based and PCR-DGGE methods. However, most isolates identified belonged to *Ascomycota* in both methods. *Coniochaetaceae* comprised the highest majority of fungi among willow cutting samples whereas *Dothioraceae* was identified as the most frequent family using the cultural method in 2007. The results of willow cutting sample analyses were nearly consistent with the results of the culture-based method except for yeast-like *Kabatiella microsticta* which showed a decrease (by 4.38%) in the frequency of isolated fungi using the PCR-DGGE method. This could be due to the fact that PCR-DGGE analysis was probably less sensitive than plate culture for describing the diversity of all fungal populations including *Kabatiella* producing yeast-like conidia. This situation has been reported in investigations of yeast species and yeast-like fungi on grapes using cultural and molecular methods (Beh, 2007). Another reason that may be responsible is the presence of nonspecific bands in DGGE gels which clearly shows the importance of what primer sets were used. The selection of appropriate primer sets is critical because the results of fungal DGGE may be affected by the targeted rDNA regions and the length of the targeted sequences (Hoshino and Morimoto, 2008). However, *Lecythophora* sp. and *Glomerella cingulata* showed an increased frequency using DGGE compared to culture-based method by 11.4% and 6.8 % respectively (Figure 3-11) which suggest more scientific evidence is required to identify the role of other factors in fungal biodiversity analyses using these techniques.

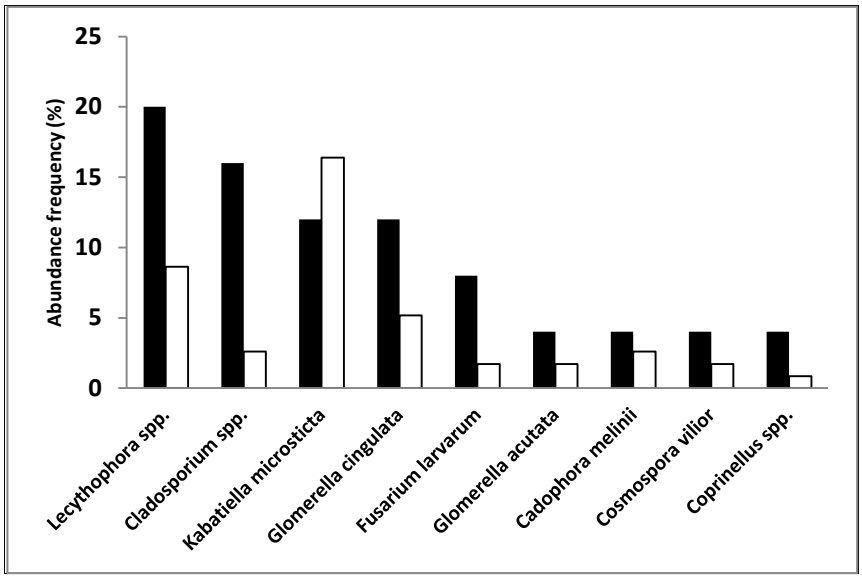


Figure 3-11. Abundance of fungal species in willow cuttings; (□) as determined by culture-dependent/PCR amplification and (■) as determined by DGGE methods (DGGE analysis is based on percentage of all bands detected).

3.6.2. Stem/Bark and Leaf

In our study, profiling the bark and leaf fungal communities of grown willow trees, we found 106 fungal sequences that belonged to a widely diversified range of fungi. Based on cloned 18S rDNA gene sequence analysis, fungal communities varied substantially between healthy and diseased plants.

According to the results, *Pleosporales*, followed by *Diaporthales* were the main fungal organisms' inhabiting diseased stem and leaf samples among Canadian willows. It was stated that *Cryptodiaporthe salicella* (*Diaporthales*) was the most important canker causing fungus in Swedish biomass willow plantations (Åström and Ramstedt, 1994). However, our findings indicated that species such as *Cytospora* spp. and *Alternaria* spp. were the most abundant isolates among diseased willows in Canada. These results also indicate that even though many fungal taxa were isolated from diseased willows, only a few of them were primarily pathogenic.

Cytospora spp. and related teleomorphic species such as *Leucostoma* and *Valsa* are not considered as active pathogens for willow, and they usually attack trees which suffer from water stress, causing Cytospora canker (Hubes, 1983). *Cytospora* canker is a fungal disease that attacks many trees species in the urban forests and orchards. This fungus is usually associated with open canker in wounded or stressed bark in hardwood plants such as in Canadian peach orchards (Buck et al., 1998). This fungus was recorded as among the most common isolated fungi for canker and dieback among Iranian willow specimens (Fotouhifar et al., 2010). *Cytospora chrysosperma* (teleomorph: *Valsa sordida*) was separated in willow stems in Alaska (Furniss, 2004) and Quebec (Vujanovic and Labrecque, 2002). *C. chrysosperma* was also isolated from aspen and cottonwood in the US as a pathogenic fungus in these tree species (Kepley and Jacobi, 2000). The susceptibility of willow trees to *C. chrysosperma* infection has been reported, especially in unfavourable conditions, such as severe winters causing frost damage, poor sites, long periods of drought, mechanical injury, damage by root-feeding nematodes and other insects, and damage or infection by other pathogenic fungi (Rawat et.al, 2006). As this fungus was isolated from stem/leaf willow tissues in our study, the possibility of disease transmittance from one tree species to another should be considered as a serious threat for distribution of fungal diseases in willow plantations.

Isolation of *Alternaria* from both diseased and healthy samples in most clones indicated the importance of their role as a general saprophyte or facultative parasite in different conditions.

Although most *Alternaria* species are common saprophytes for plants, some of them are plant pathogens that cause important diseases like stem canker, leaf blight or leaf spot (Thomma, 2003). The isolation of *Alternaria* spp. in healthy leaves of *Salix* spp. (Vujanovic and Labrecque, 2008) demonstrates their potential role as beneficial fungi.

Some isolated fungi such as *Glomerella* spp., *Sclerotinia* spp., *Botrytis byssoidea, Monilia laxa, Marssonia populi*, *Diaporthe eres, Persiciospora africana, Valsella* spp. and *Valsa* spp. were mostly identified in diseased stem and leaf samples in this study. This may suggest that the colonization of these fungi occurred more in diseased plants under specific conditions. Vujanovic and Labrecque in 2008 reported several fungal taxa from willow plants. Most of the isolated fungi such as *Valsa* spp., *Venturia* spp., *Glomerella* spp., *Epicoccum* spp., *Alternaria* spp., and *Marssonia* spp. belonged to *Ascomycota* (Vujanovic and Labrecque, 2008) which supports our finding regarding isolated fungi in willow aerial tissues.

Fungal profiles in healthy stems and leaves included many important species, particularly in the areas of plant pathology and plant systematic. Among them there were some fungi which can be considered as potential antagonists. We found that some fungal isolates such as *Humicola fuscoatra, Pseudodiplodia* sp., *Fimetariella rabenhorstii, Chaetomium globosum, Cladosporium* spp., *and Microdiplodia* sp. were exclusively found in healthy stems or leaves, which could imply that they have the potential to compete with pathogenic fungal groups as natural antagonists. It may indicate that they have competed with the pathogens and reduced their ability to invade and colonize willow clones (Woo and Lorito, 2007). Among the identified fungal taxa, there were several fungal endophytes in healthy stems and leaves which could have beneficial effects for the plant in controlling pathogenic fungi. Therefore, additional data on the fungal biodiversity associated with willow plantations is crucial to prevent and manage the unwilling introduction of new foreign fungal pathogens (Crous. 2005).

Based on biodiversity indices, the fungal communities related to diseased samples were more diversified than on healthy samples, which indicate a probable multi-factorial nature of pathogenic fungi in disease occurrences. Moreover, it suggests that the health status of plants can influence fungal taxa assemblages. In addition, the results of this study indicated that fungal communities were varied depending on plant organs (stem/leaf). Diversity indices were higher in healthy leaves compared to healthy stems, and on the contrary, the indices were higher in diseased stems in comparison with diseased leaves. High level of diversity in the Alberta

plantation may be due to several reasons including differences in soil texture and climatic conditions. The results clearly show that some fungal species depicted a degree of plant organ specificity. As evidence, *Marssonia populi, Glonium pusillum, Monilinia laxa, Anthostomella conorum, Apiospora montagnei, Pithya cupressina, Trichoderma longibrachiatum* were observed only in diseased leaves. On the other hand, *Valsa* sp., *Ceratobasidium* sp., *Leptosphaerulina trifolii, Diaporthe fibrosa, Glomerella acutata, Glomerella cingulata, Persiciospora africana, Aureobasidium pullulans, and Botrytis byssoidea* were isolated only from diseased stems. However, more supporting evidence is needed to clarify the specificity of isolated fungi in different organs.

The composition of willow stem/bark and leaf fungal communities may be influenced by sampling site characteristics and geographic locations. For instance, the fungal communities from Alberta, Manitoba, and SK1 with dominance of *Alternaria* spp., was different from SK2 with dominance of *Cytospora* spp. as potentially pathogenic species. Differences among fungal communities in different sites were previously reported to be associated with willow leaf/bark (Vujanovic and Labrecque, 2008) and root (Fujimura, 2007; Corredor, 2011). The pathogenic fungus *Marssonia salicicola* was isolated from weeping willow in Montreal area, Canada (Vujanovic et al. 1998; Vujanovic and Labrecque, 2002). However, we isolated *Marssonia populi* in our study. Moreover, the previous crop could influence the fungal biodiversity in different willow plantation locations. For instance, in Manitoba site previous crop mentioned as shallot while in other locations it was cereals.

Athought the *Glomerella miyabeana* pathogen was not detected, which is a cause of black-canker in various willow species in New Zealand (Spiers and Hopcroft, 1993), we found frequently *Glomerella cingulata,* anamorphic *Colletotrichum gloeosporioides*, as well as *G. acutata* species throughout western Canadian provinces. Previously, *Glomerella cingulata* has been reported in eastern Canada on willow green wall in urban environments (Vujanovic and Labrecque, 2008). These fungal taxa may be primary pathogens for willow under stressful environmental conditions. However, *Glomerella cingulata* and *G. acutata* were often reported on agriculture crops (Sutton 1992). Hence, the agricultural history of the sites and preceding (wheat, barley, oats, pulses, canola etc.) crops could influence the occurrences of different fungal communities trough out sampling sites. This can raise the question whether the fungal pathogens

from preceding plant hosts can jeopardize the willow health and/or biomass production under SRIC.

Melampsora spp. as obligate fungal pathogens and the cause of willow rust disease (Pei et al., 2010; Dawson, 2007) were not present in the Canadian prairies. The reason behind this could be the existence of natural anthagonists which inhibit this pathogen's attack or prevent its distribution. Severe climate and frequent frost injury events are other factors controlling the existence of different fungal pathogens in willow plantations in various geographical regions (Ramstedt et al., 1994).

Variations in susceptibilities to diseases have been reported in different willow clones (Hunter 1996, Labrecque 2005). As evidence, clones obtained from *S.caprea* and *S. aurita* were identified as more susceptible to *Melampsora caprearum* (Hunter, 1996). Clones *Hotel, Juliet, SV1*, and *India* are naturally adapted for biomass production in North America (Kopp, 2001). The presence of particular fungal populations on different clones was also recorded in this study. For example, low fungal diversity was identified in healthy leaf and diseased stem samples of the clone *SX64* whereas the low diversity was found among clones *Hotel* and *SX61* in healthy stem and diseased leaf samples. This situation may be related to genetic characteristics of different clones (Rönnberg-Wästljung, 2008) including fungus-host compatibility relationships.

Comparison of fungal communities using DGGE and culture-based methods indicated that family *Trichocomaceae* (*Aspergillus niger* isolate 6) was found by the DGGE method but was not isolated using the culture method from the same sampling sites (SK2 and Alberta). The majority of fungal isolates from stem/leaf samples belonged to the families *Davidiellaceae* and *Valsaceae,* at the University of Saskatchewan willow plantation, according to the results of DGGE. However, the results obtained from cultural methods revealed that *Valsaceae and Pleosporaceae* were the abundant isolated fungi while *Davidiellaceae* family belonged to a less frequently encountered fungal group. In total, fungal inhabitants' patterns for Alberta willow plantation was almost the same in both methods with the abundance of family *Pleosporaceae.*

Many fungal species such as *Cytospora chrysosperma, Leucostoma persoonii, Valsa* spp., *Valsella* spp., *Lewia infectoria, Alternaria* spp., *Davidiella macrospora, Arthrinium* spp., *Sclerotinia* spp., *Marssonia populi, Coprinellus* spp., and *Phoma* spp. commonly isolated using cultural methods were identified easily using the DGGE approach. This method revealed that fungal species including *Cytospora chrysosperma, Davidiella macrospora*, and *Lewia infectoria*

exhibited increased frequencies compared to the culture-based method. However, *Alternaria* sp. showed considerably decreased frequency in environmental samples by 10.8%, probably due to lower sensitivity of PCR-DGGE compared to plate culture, or due to nonspecific bands in DGGE gels (Hoshino and Morimoto, 2008) (Figure 3-4). Besides, undefined specific boundaries for some genera of fungi have been stated to explain difficulties in developing an accurate identification using the DGGE method (Mavragani, 2008). This study demonstrated that DGGE can be used to characterize different fungi in environmental samples and that the culture-based approach is sometime insufficient to define biodiversity (Muyzer et al., 1993). As evidence, fungal isolates such as fungal endophyte isolate 9055, *Cadophora luteo-olivacea* strain 7R38-4, and *Cosmospora vilior* isolate olrim557 recovered from DGGE sequence analyses indicated that these fungi were possibly uncultivable or slow growing on media using the culture-based method.

There is limited information of fungal diversity, ecology and functions in willow SRIC plantations in North America (Vujanovic and Labrecque 2002). Classification of fungal isolates in functional categories is difficult because of overlap in their behaviors regarding the fungal life cycles (sexual and asexual stages), climatic and environmental conditions, or plant organ and growth stage (Arnold, 2007). Our results support the hypothesis that fungal taxa fluctuations may be related to fungus-fungus and fungus-host genotype interactions, which could affect a willow's health and its susceptibility to disease agents (Vujanovic and Labrecque, 2008). However, further research is necessary to fully evaluate fungal community structures with a possibility to control pathogens under different environmental conditions in respect of the willow stress tolerance changes over time under SRIC-short rotation system.

4. ABOVE-GROUND BIOMASS PRODUCTION AND RELATIVE RATE OF PHOTOSYNTHESIS IN WILLOW GENOTYPES GROWN UNDER SRIC

4.1. Abstract

Utilization of short-rotation intensive cultural (SRIC) willow systems is being developed as a source of bioenergy in North America. Photosynthetic activity has an important role to play in biomass production. To measure photosynthesis, seven different genotypes or clones from a three-year-old willow plantation belonging to the University of Saskatchewan were studied over the willow growing season. There was a significant difference between the average rates of photosynthesis in healthy vs. diseased willow leaves. The highest photo-activity was recorded in healthy *Charlie* and *SV1* clones. High biomass production is associated with several factors such as stem diameter, the number of stems, and wood density. In this study, the stem diameter was assessed to estimate willow biomass production. Based on this method, we were able to identify the differences in productivity between healthy and diseased willows. Data analyses showed that most of the willow clones had a similar biomass composition; however, there was a significant difference between healthy and diseased samples. Clones *Charlie* and *SV1* exhibited the highest biomass production in both healthy and diseased samples. These results are valuable in assisting in the selection of the most effective *Salix* genotypes for the purpose of disease resistance and SRIC sustainability.

4.2. Introduction

The gradual depletion of fossil fuel resources and global environmental concerns has led to the growth of interest in the utilization of renewable sources of energy. Bioenergy from biomass can be considered as an important tool to reduce greenhouse gas emissions and as a means of sustainable energy supply (Berndes et al., 2003; Sims et al., 2006)**.** The term biomass refers to any solid or nonsolid biological energy source. SRIC (short-rotation intensive culture) is defined as a fast-growing, high-yielding woody crop that is managed under a coppice system and harvested every two to four years (Zeller et al., 2009). This length of the rotation is considered as an important factor for the production of short rotation species and for producing high levels of biomass (Picchi et al., 2006). Willow (*Salix* spp.) is the most common woody plant that has been widely used as a source of bioenergy in the northern hemisphere (Larsson et al., 2003). The first

willow SRIC for biomass production was established in Northern Europe and in the United States several years ago (Volk et al., 2004).

Plant productivity relies on the interactions between the absorption of light and CO_2 by leaves. Differences in the amount of leaves and subsequently in the intensity of photosynthesis result in variability in biomass production. Photosynthetic activity is also different among trees. For instance, deciduous trees have higher photosynthetic rates than coniferous trees (Barigah et al., 1994). Similar to plant growth, photosynthesis rate is directly related to biomass production (Picchi et al., 2006). Furthermore, leaf area plays a key role in biomass productivity. Variability in plant genotypes and phenotypes, such as plant branching patterns, and leaf distribution and position, control the rate of photosynthesis and subsequently the biomass productivity of the tree (Barigah et al., 1994). Any changes in plant health status can alter photosynthetic activity and also adjust microbial community structure (St-Arnaud and Vujanuvic, 2007). The measurement of stem diameter may allow one to judge the status of diseased and healthy willow plants.

4.3. Objectives and Hypothesis

The hypothesis underlying this study was that diseased willows have lower photosynthesis performance and subsequently less biomass production than healthy plants. The objectives of this study were to compare biomass estimation using stem diameter with measurements of photosynthesis in willow plants, and to evaluate the effects of plant health status on biomass production and photosynthetic activity in different willow clones.

4.4. Materials and Methods

4.4.1. Study Design

4.4.1.1. Photosynthetic Activity Measurement

The present experiment was designed to measure the photosynthetic activity in seven clones of a three-year-old willow plantation over a period of an active growth of willow (between June 15 and July 15, 2009). Chlorophyll fluorescence was determined with a portable chlorophyll fluorescence measurement system (model OS-30P- Opti-Science; Hudson, NH, USA). The value of photochemical efficiency Fv/Fm (variable fluorescence to maximum fluorescence) was recorded on 42 healthy and diseased unfolded leaves with three replicates per clone (Belkhodja et al., 1994; Ögren and Rosenqvist, 1992). These measurements were carried out between 9 and 11

am. A leaf clip was attached to the upper surface of the leaf and a shutter plate was closed, so that the leaf adapted to the dark. The darkness adaptation time lasted for 20 minutes (satisfactory adaptation period) before a measurement was taken. Fluorescence was recorded at 650 nm following manufacturer's instructions.

4.4.1.2. Biomass Measurement

Biomass measurement as a conventional allometric technique (defined by measurement of stem diameter) was used in this experiment. 42 randomly selected willows (half of them healthy and half of them diseased plants) were collected from a 4-year-old hybrid plantation belonging to the University of Saskatchewan. This plantation consisted of three blocks and each block was divided into seven clones. Three repetitive measurements were made for each sample. In order to determine the above-ground biomass, the central stem diameters were measured at a height of 30 cm with a mechanical calliper according to the formula: $Y=eD^{a}$ where Y is willow biomass, e is the regression equation, D is stem diameter (mm), and *a* is an exponential factor (Ballard 1998, Arevalo 2007, Hangs, personal communication).

4.4.2. Statistical Analyses

Statistical analysis was performed using SPSS version 10 (SPSS Inc., 2000). Data were tested for normality by use of the Shapiro-Wilk W-test for goodness of fit, and the equality of variances was tested by use of the Levene test. When the variances were equal, one-way analysis of variance (ANOVA) was used for separation of mean difference values of photosynthetic and willow biomass measurements. When the variances were not equal, the nonparametric Kruskal-Wallis test was performed. Comparison between healthy and diseased samples was performed using Paired Sample T-test. When there was not normal distribution, then Wilcoxon Signed Ranks Test was used. Values of $P<0.05$ were considered significant for differences among groups for all tests at 95% confidence interval.

4.5. Results

4.5.1. Photosynthetic Activity in Healthy and Diseased Willows

The mean of photosynthetic measurements (PM) was equal to 0.73 ± 0.02 µmol/m² for diseased willows compared to 0.75 ± 0.03 µmol/m² for healthy willows (Figure 4-1). According

to the statistical analyses, there was a significant difference between the two means (paired t-test; $p=0.02$) (Figure 4-1). There was also a significant difference within the variance of groups (clones) in healthy willows (ANOVA, $p=0.002$) while no significant difference was recorded for the diseased group (Kruskal-Wallis Test, $p>0.05$).

Among healthy willows, clone *Charlie* had the highest PM value (0.81 ± 0.01 µmol/m²), followed by *SV1* (0.79 ± 0.01 µmol/m²). However, clone *India* had the highest recorded PM value in diseased willows (0.77 ± 0.02 µmol/m²). Clone *SX64* in diseased willows had the lowest PM value (0.55 ± 0.15µmol/m²) (Figure 4-2).

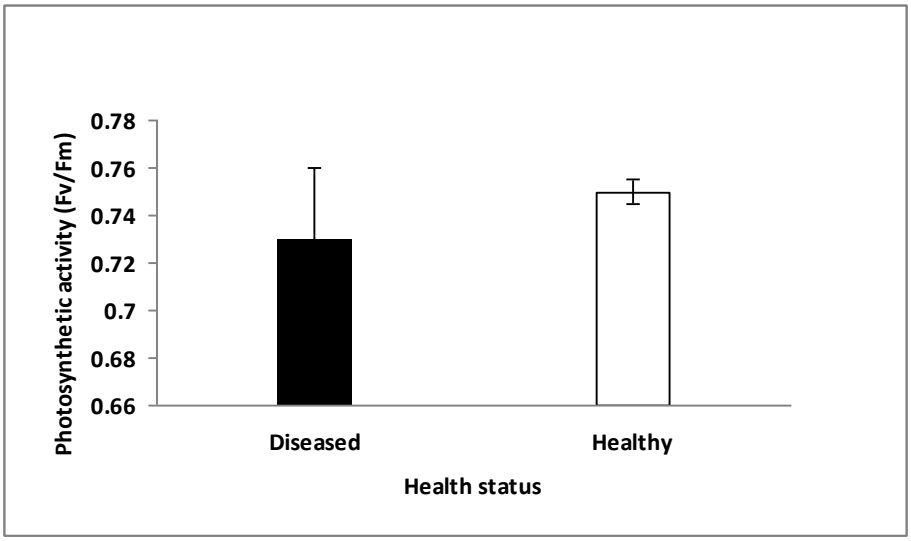


Figure 4-1. Difference in photosynthesis (Fv/Fm) ratio in healthy (□) vs. diseased (■) willow leaves

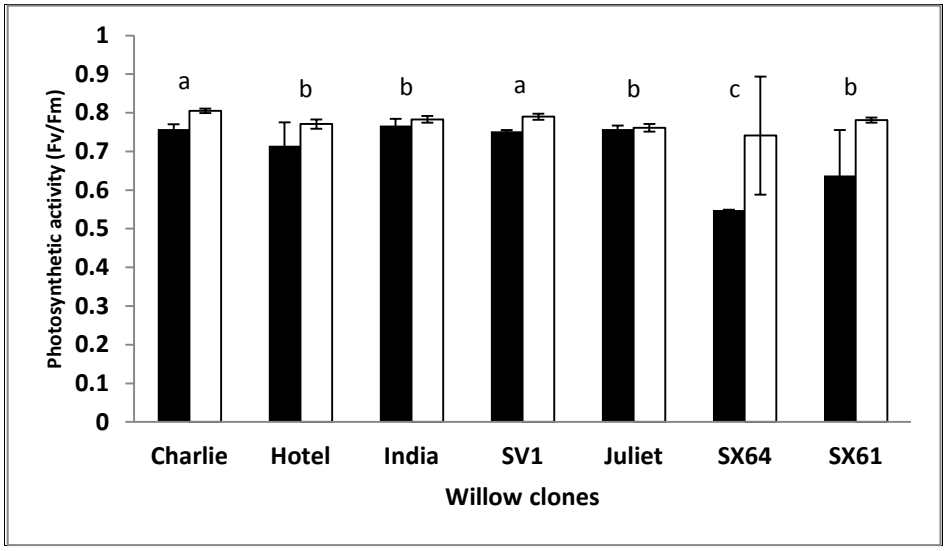


Figure 4-2. Comparison between photosynthesis (Fv/Fm) ratio related with healthy (□) vs. diseased (■) status of different willow genotypes grown in the University of Saskatchewan clonal plantation.

(Mean values followed by the same letter were significantly different from others according to ANOVA test at $P < 0.05$).

4.5.2. Biomass Production

The total mean of produced biomass was 211.8 ± 25.5 kg/ha in healthy samples and 110 ± 13.8 kg/ha for diseased willows (Figure 4-3). Among all clones, the mean of biomass for the fourth–year plants ranged from 72.0 ± 1.0 kg/ha for clone *Juliet* to 246.0 ± 12.4 kg/ha for clone *Charlie* in diseased plants and from 138.7 ± 11.6 kg/ha for clone *SX61* to 407.0 ± 15.0 kg/ha for clone *Charlie* in healthy plants. The results indicated that clones *Charlie* and *SV1* had superior performance in biomass production in both healthy and diseased samples (Figure 4-4). There was a significant difference between the means of biomass levels of healthy and diseased plants (Wilcoxon Signed Ranks Test, $p< 0.05$). However, there was a significant difference within the variance of groups (clones) (Kruskal-Wallis Test, $p< 0.05$) in both healthy and diseased treatments (Figure 4-3).

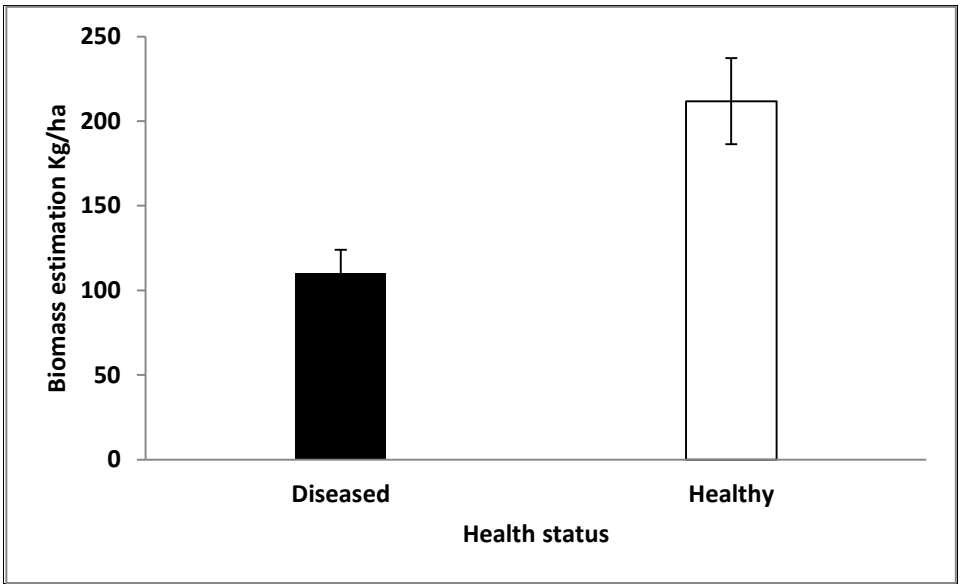


Figure 4-3. Comparison between total biomass production in healthy (□) vs. diseased (■) willow

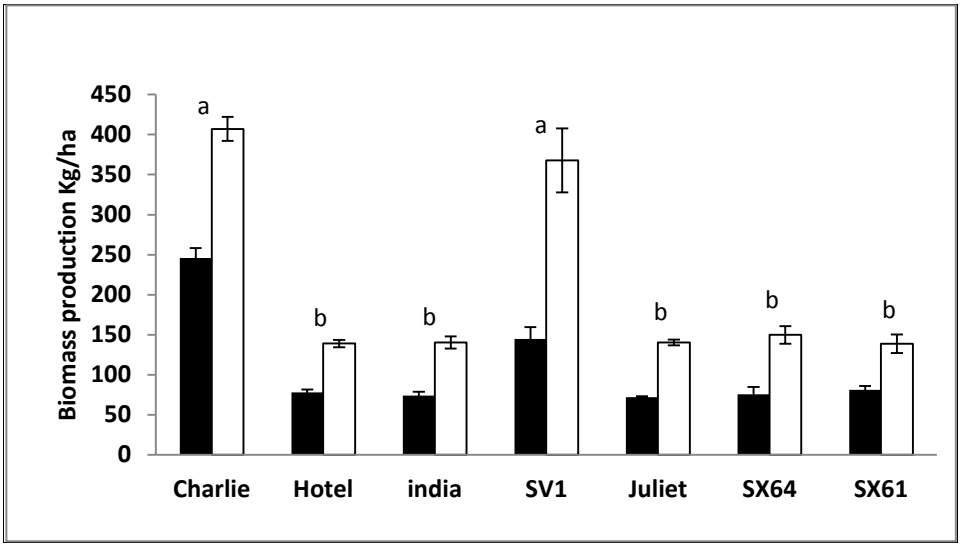


Figure 4-4. Differences in biomass production over a growth season in healthy (□) and diseased (■) willow genotypes from the University of Saskatchewan clonal plantation. (Mean values followed by the same letter were significantly different from others according to Kruskal-Wallis test at $P < 0.05$)

4.6. Discussion

In this study, we looked at biomass productivity by measuring stem diameter, and compared the values for both healthy and diseased willow in the four-year-old multi-clonal plantation. Stem diameter measurement was proposed as an efficient method to estimate the willow biomass production under SRIC (Ens et al., 2009). Our results indicated that the average estimated biomass for diseased samples was significantly less than the mean biomass for healthy samples. The best willow biomass productivity was obtained from clones *Charlie* and *SV1* and the least was recorded for clone *Juliet*.

Our results indicated that the highest biomass was recorded in clone *Charlie* and *SV,* whereas the least was recorded for clone *Juliet* among healthy willows. This could point out the importance of appropriate selection of the willow species in biomass production (Sims et al., 2001). However the results of fungal biodiversity showed the highest biodiversity indexes (Shannon's diversity) for clone *India* (in healthy plants), which was not consistent with the results of biomass production (stem diameter). This suggests that the impact of other factors, such as age of root stock, genotype adaptability and compatibility with beneficial fungal communities, can affect the willow biomass production (Sims et al., 2001). The biomass production values can be result of many other interrelated factors (Verwijst and Telenius, 1999; Bungart and Huttle, 2001) such as variability in soil characteristics, water availability or weather conditions, usage of herbicides or fertilizers (Bergkvist and Ledin, 1997; Tahvanainen and Rytkönen, 1999) and density of the willow plantation (Bergkvist and Ledin, 1997; Adegbidi et al., 2001). According to Bergkvist and Ledin (1997) and Corredor (2011) the root system and rhizophere microbial activity could also influence the efficiency of plant growth, the biomass production and associated photosynthetic activity (Bergkvist and Ledin, 1997; Corredor 2011).

In the present study, photosynthetic activity was determined to be different in healthy and diseased willow leaves. Evidence was presented that the decline in Fv/Fm ratio was observed in diseased leaves. These results are in agreement with the literature, which has shown that infections of willow leaves, such as willow rust disease, leads to decreased photosynthesis due to less chlorophyll being present (Abd et al., 2009; Pei et al., 2003; McCracken and Dawson, 1998). In addition, different photosynthetic activities may be due to the changes in growing seasons and distribution of photosynthetic rates within the trees. Evaluation of photosynthesis

activity in different clones indicated that Clones *Charlie* and *SV1* were the best performing clones with high photosynthesis rates. Clone *SV1* has been particularly investigated in the United States as a standard clone for biomass production in SRIC plantations (Kopp 2001). The low mycodiversity and consequently low susceptibility to pathogenic fungal colonization in *SV1* (Baum and Hrynkiewicz 2006) could justify superior photosynthesis activity in this clone. However, this is not true for clone *Juliet* which has the same parental species (*S. dasyclados*) with *SV1*. Therefore, the role of other factors regarding photosynthetic activity must be considered. No reports were found about the origin of the clone *Charlie* and its performance in SRIC. A positive relationship between biomass production and photosynthesis activity has been demonstrated in larch (*Larix* spp. Family: *Pinaceae*) (Matyssek and Schulze, 1987). However, in some cases, such as *Populus grandidentata*, photosynthetic rate had a poor correlation with the biomass production of the plant (Barigah et al., 1994; Reighard and Hanover, 1990). In this study, we found a positive relationship between photosynthesis activity (Fv/Fm) and woody biomass production (based on stem diameter measurement) in both healthy and diseased willows related with *Charlie* and *SV1*- the most productive clones. The highest productivity of these clones is probably in accordance with their large leaf area index and subsequently photosynthetic performance (Merilo et al., 2006). Furthermore, the maximum above-ground biomass productivity was positively related to their high photosynthetic rates. Clones *SX61* and *India* had relatively high photosynthetic rates and a low biomass production compared to other clones. This could probably be due to the effects of other factors on aboveground biomass production and their leaf size and expansion (Sims et al., 2001).

5. EFFECTS OF POTENTIALLY PATHOGENIC AND BENEFICIAL FUNGI ON ABOVE-GROUND TISSUES OF WILLOW

5.1. Abstract

The effects of potentially beneficial and pathogenic fungi, inhabiting willow aerial tissues, were evaluated using apple-based *in vitro* and greenhouse *in vivo* assays. The results of the pathogenicity test in an apple bioassay indicated the potential pathogenic capacities of *Truncatella angustata, Glomerella cingulata, Sclerotinia* sp.*, Protoventuria alpina, Leucostoma niveum, Diaporthe eres,* and *Cadophora luteo-oliviace*. The results from the direct inoculation of the fungi on willow cuttings showed infection percentages ranging from 13.7% ± 7.0 caused by *Botrytis byssoidea* to 79.7% ± 10.4 caused by *Glomerella cingulata* in willow leaves. Furthermore, severe open cankers were observed with the presence of *Leucostoma niveum, Cytospora chrysosperma, Valsa salicina,* and *Valsella melostoma* on willow stems four weeks after inoculation. Potentially beneficial isolated fungi from willow samples were studied by dual-culturing in the presence of the phytopathogenic fungi: *Glomerella cingulata, Fusarium gruminarum,* and *F. avenaceum*. Among 12 promising beneficial fungi tested, three of them: *Lecythophora* sp.*, Kabatiella* sp.*,* and *Coprinellus* sp. illustrated an ability to suppress the growth of pathogens. Screening of these fungi revealed that there was wide variation among these isolates with regards to their colonization and inhibition behavior in plate cultures, but none of the fungi demonstrated total inhibition of the growth of pathogens.

5.2. Introduction

Fungi are among the most common pathogenic threats for various trees such as the willow species (Mueller and Schmit, 2007). For example, members of the *Valsaceae* family including; *Valsa salicina, Leucostoma niveum,* and *Cytospora chrysosperma* are the cause of stem canker disease, but can also initially infect leaves and subsequently cause stem lesions systematically (McCartney et al., 2003). The occurrence of fungal stem cankers caused by *Valsaceae* has been the most significant disease problem in European countries, along with leaf rust associated with *Melampsora* spp., and frost die-back caused by *Peseudomonas syringae* rot (Ramstedt et al., 1994).

Natural methods of disease control are considered to have several benefits for willow short rotation intensive culture (SRIC) and biomass production. Information about the beneficial/ antagonist fungi is relatively limited (Butt and Copping, 2000). A number of studies have reported that the beneficial plant-associated fungi may affect plant growth and enhance resistance to diseases. For example, mycorrhizal fungi are considered as biocontrol agents, biofertilizers, and growth stimulators in agriculture (Compant et al., 2010). In the recent decade, many microbial biocontrol agents have been reported to be effective in preventing many plant diseases. The biocontrol abilities of beneficial fungi against pathogens include secretion of antibiotic-like substances, induction of host resistance, or competition for nutrients or space; these can lead to increased plant protection (Mercier and Lindow, 2000; Jones and Prusky, 2002). In particular, induced tolerance can be a very important biocontrol and beneficial factor in plant tissues in the presence of environmental stresses (Sequeira. 1983; Janisiewicz et al., 2008). A number of studies have focused on isolation, characterization, and use of new biocontrol fungal organisms; however, the effects of these beneficial microorganisms on non-target tissues such as plants are not well-known yet. Recently, the increase in resistance against chemical fungicides, the presence of environmental residues, and public concerns about the use of artificial chemicals has led to emphasis on recognizing and developing new biocontrol fungal microorganisms. For example, effects of a potential biocontrol agent of apple powdery mildew (*Podosphaera leucotricha*), and scab infection agent (*Venturia inaequalis*) were examined on the host plant by inoculation of the pathogen into fruit tissue (Alaphilippe et al., 2008). Advantageous fungal species on healthy willow plants can be influential agents for controlling the existence of pathogenic fungi; however, our knowledge related to such microbial communities is narrow and not profound. Recognition of fungal diversity would be important for the detection of pathogenic isolates and prevention of their establishment in willow plantations (Butt and Copping, 2000).

5.3. Objectives and Hypothesis

The hypothesis underlying this study was to evaluate the functional effects of potentially beneficial and pathogenic isolates from above-ground willow tissues grown under SRIC systems. The purpose of this study was: *i)* to develop a reliable comparison system for identification of the potentially pathogenic fungi isolated from above-ground willow tissues, and

ii) to characterize and identify the antagonistic capacity of the isolated fungal species against different fungal phytopathogens in dual culture assays.

5.4. Materials and Methods

5.4.1. Fungal Pathogenicity Potential

5.4.1.1 Apple Bioassay

The pathogenicity of potentially pathogenic fungi (Table 5-1) was evaluated using an apple-based *in vitro* test (Vikram et al., 2004; Badosa et al., 2009). Healthy mature golden delicious apples (*Malus domestica* Borkh.) were surface-disinfected using a sterile tissues and ethyl alcohol (95%). Four holes (1-2 mm diameter and about 5-6 mm depth) were made around the equatorial region on each apple by a cork borer (Henriquez, 2005). Twenty µl of fungal suspension was inoculated using a micro pipette on those apples. An inoculation with sterile water was considered as a negative control on each apple. Three replicates were considered for each fungal isolates and the assay was repeated twice. Apples were placed in water-sprayed plastic bags to provide a humidity-saturated atmosphere and kept at room temperature and in the dark. The lesion diameters were measured after 10 days of fungal inoculation.

Table 5-1. Potentially pathogenic and beneficial fungi tested using apple-, greenhouse- and dual-culture assays

OTU	SMCD	BLAST match	Anamorph/ Teleomorph	Accession No.	Similarity (%)	Origin	Dual-culture	Apple	Greenhouse
34.1	2520	*Botrytis byssoidea*	*Botryotinia*	FJ169671.1	99%	leaf			√
124.3	2611	*Cadophora luteo-olivacea*	NA	GQ214536.1	98%	Cutting	√	√	
119.4	2616	*Cadophora melinii*	NA	DQ404351.1	97%	Cutting	√	√	
120.5	2621	*Choniochaeta veluntina*	*Lecythophora*	FJ167402.1	100%	Cutting		√	√
126.3	2617	*Cladosporium cladosporioides*	*Davidiella*	AY251074.2	99%	Cutting	√	√	
125.1	2623	*Coniothyrium* sp.	*Leptosphaeria*	EU770235.1	97%	Cutting	√		
122.4	2624	*Coprinellus* sp.	NA	EU436684.1	99%	Cutting	√		
103.3	2625	*Cosmospora vilior*	*Acremonium*	FJ824628.1	95%	Cutting	√	√	
32.3	2534	*Cytospora chrysosperma*	*Valsa*	FJ478104.1	99%	stem		√	√
118.3	2627	*Diaporthe eres*	*Phomopsis*	FJ478132.1	98%	Cutting		√	√
*	2248	*Fusarium avenaceum*	*Gibberella*	NA	NA	SMCD		√	√
*	2241	*Fusarium culmorum*	*Gibberella*	NA	NA	SMCD			√
*	2243	*Fusarium graminearum*	*Gibberella*	NA	NA	SMCD		√	√
101.2	2649	*Glomerella cingulata*	*Colletotrichum*	AJ301952.1	99%	Cutting		√	√
101.4	2655	*Kabatiella microsticta*	*Discosphaerina*	EU167608.1	99%	Cutting	√	√	
103.2	2661	*Lecythophora luteoviridis*	*Coniochaeta*	DQ404354.1	96%	Cutting	√	√	
25.6	2565	*Leucostoma niveum*	*Valsa*	AF362558.1	99%	stem		√	√
112.2	2674	*Phialocephala* sp.	NA	FJ903362.1	100%	Cutting	√		
104.1	2677	*phoma glomerata*	*Alternaria*	AB470828.1	98%	cutting	√		√
111.3	2682	*Protoventuria alpina*	NA	EU035444.1	99%	Cutting		√	√
111.4	2683	*Rosellinia nectrioides*	NA	FJ175181.1	98%	Cutting	√		
12.7	2594	*Sclerotinia* sp.	*Botryotinia*	AJ279480.1	99%	leaf		√	√
120.4	2686	*Truncatella angustata*	*Broomella*	AF377300.1	98%	Cutting	√	√	√
15.10	2601	*Valsa salicina*	*Cytospora*	EF447364.1	99%	stem		√	√
32.6	2604	*Valsella melostoma*	NA	AF191184.1	98%	stem		√	√

* Saskatchewan Microbial Collection Database, SMCD, Saskatoon, Sk.

5.4.1.2. Greenhouse Test

The greenhouse experiment was conducted at the University of Saskatchewan greenhouse using 15 fungal isolates (Table 5-1) on *Hotel* cultivar, which belong to the *Salix purpurea* - a dominant willow species grown in western Canada. At the time of growing the first buds, inoculation with fungal isolates was started. This experiment was performed twice and each time it contained three replications per treatment. Pure fungal cultures were prepared in Potato Dextrose (PD) broth at (28 ºC ± 2) using a rotary shaker (140 rpm) until a suspension of mycelium-producing units (10^5 units mL^{-1}) was obtained. 500 µl of fungal suspension using a micro pipette was sprayed on the young willow buds and then rubbed in using a sterile swab without damaging the young leaves. To test stem fungal pathogens, 25 µl of fungal suspension was inoculated on the young stems (2-3 cm) by cutting a T-shaped scar (12×10 mm) using a sterile blade. Sterile water was used as a negative control. The evaluations were started approximately two weeks after inoculation of the fungal suspensions. Disease severity as described by Vujanovic et al. (2000) was calculated for each treatment using the following formula; [Number of symptomatic bark or leaf samples / Total number of samples] × 100 (Abd et al., 2009; Tondje et al., 2007). Successful infections, including leaf and stem, were used for re-isolating fungal pathogens on PDA media satisfying Koch's postulate.

5.4.2. Fungal Antagonistic Potential

Three potentially pathogenic fungi (*Fusarium graminearum* SMCD# 2243), *F. avenaceum* (SMCD# 2241), and *Glomerella cingulata* (SMCD# 2649) were selected to evaluate the degree of hyphal reduction/inhibition reaction of some of the potentially beneficial fungi found on willow samples (Table 5-1). A 5 mm diameter plug of each fungal isolate was taken from a 7-day-old pure culture and these were placed approximately 8 cm apart on a PDA plate. The fungal pathogens were then placed on the center of each plate (Figure 5-1). Three replicates of each fungus were prepared (Harveson and Kimbrough, 2001; Li et al., 2003). After one week of incubation, percent inhibition (PI) (Figure 5-2) was calculated as: % of inhibition = (growth diameter of control) – (growth diameter of the treated sample with fungus) / growth diameter of the control ×100 (Dastager et al., 2009; Bruno and Sparapano, 2006; Radjacommare et al., 2010). As well, the overall growth of phytopathogens was evaluated through the inhibition zone (IZ) by

measuring the distance (mm) between the edge of the phytopathogen mycelium and the antagonistic fungi.

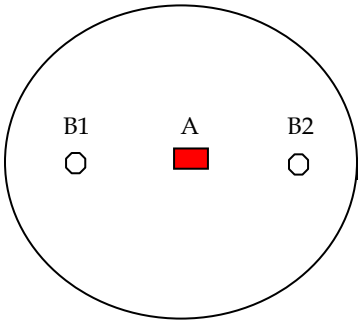


Figure 5-1. Diagram of dual-culture assay showing the fungal pathogen (*G. cingulata*, *F. graminearum*, *F. avenaceum*) colony growth inhibition by fungal antagonists in petri plates.
[Fungal pathogens (A): *G. cingulata*, *F. graminearum*, *F. avenaceum*, *G. cingulata*, and fungal antagonists (B)].

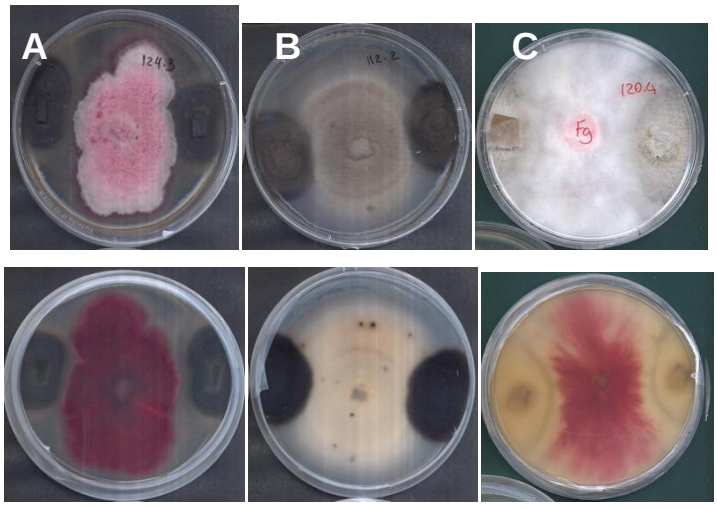


Figure 5-2. Dual culture assays of different fungal treatments against three fungal pathogens on PDA plates after 1-week of incubation.
[Above row: (A) *F. avanaceum* vs. *Cadophora luteo-olivacea*, (B) *G. cingulata* vs. *Phialocephala* sp., (C) *F. graminearum* vs. *Coniothyrium* sp. Bottom row: bottom sides of the same plates].

5.4.3. Statistical Analyses

Statistical analysis was performed using SPSS version 10 (SPSS Inc., 2000). The equality of variances was tested by use of the Levine's test. When the variances were equal, one-way analysis of variance (ANOVA) was used for separation of mean differences values. When the variances were not equal, the non-parametric Kruskal-Wallis test was performed. Values of $P \leq 0.05$ were considered significant for differences among groups for all tests at 95% confidence interval. Statistical analysis and comparison of mean of growth inhibition percentages among fungal isolates was performed by a one-way T-test.

5.5. Results

In the apple bioassay, three diagnostic bioassay categories including control (apples with sterile water inoculation), non-symptomatic (lesion diameter from 2 to 3.5 mm), and symptomatic (lesions more than 3.5 mm) was defined (Figure 5-3). The diameters of lesions were measured at 19.0 ± 1.5 mm for *Truncatella angustata* as the highest lesion diameter, followed by *G. cingulata (*15.3 ± 0.3 mm), and then *Sclerotinia* sp. (14.0 ± 1.2 mm). In addition, *Diaporthe eres, Cadophora luteo-olivacea, Leucostoma niveum, C. chrysosperma, Protoventuria alpina, F. avenaceum,* and *Cadophora melinii* induced considerable necrotic damage compared to the control ($P < 0.05$). On the other hand, *Cladosporium cladosporioides, Lecythophora luteoviridis, Valsa salicina, Valsella melostoma, Kabatiella microsticta, Choniochaeta veluntina,* and *Cadophora melinii*, did not produce significant phytotoxicity symptoms ($P > 0.05$) or caused minimum lesion size (Table 5-2 and Figure 5-4). The least brown rot lesion diameter was recorded for *Cosmospora vilior* (2.3 ± 0.3 mm).

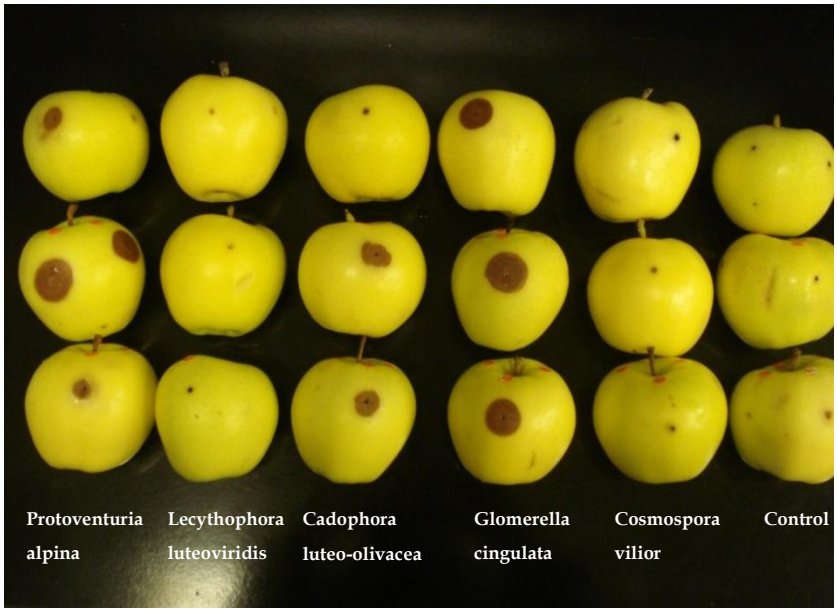


Figure 5-3. Symptomatic apple-fruits showing necrotic reactions caused by artificial-inoculation of different pathogenic fungi tested after 1-week of incubation.

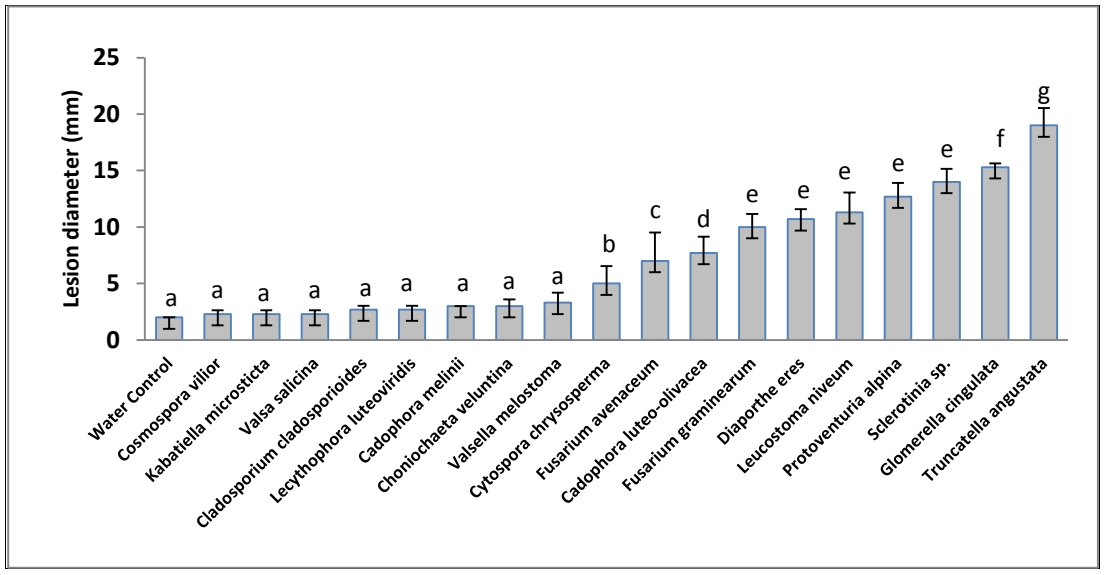


Figure 5-4. Comparison of size (mm) of the necrotic lesions caused by potentially pathogenic fungi tested using the apple bioassay.

[Values are mean ± standard error (SE) for each treatment. Treatments followed by the same letter are not significantly different from respective untreated control according to Kruskal- Wallis test at $P < 0.05$].

Table 5-2. Results on fungal pathogenicity tested in apple and greenhouse bioassays, and antagonistic activity in dual-culture assay.

OTU	SMCD	Treatment	Apple	Greenhouse	Dual-culture					
			Lesion Diameter (mm)	Infected Leaves (%)	Radial Growth Inhibition (%)			Inhibition Zone Diameter (mm)		
					F.g	F.a	G.c	F.g	F.a	G.c
–		Sterlie water (control)	2.0 ± 0.00 a	0.00 ± 0.00 a	–	–	–	–	–	–
34.1	2520	*Botrytis byssoidea*	–	13.7 ± 6.98 b	–	–	–	–	–	–
124.3	2611	*Cadophora luteo-olivacea*	7.7 ± 1.45 d	–	29.4 ± 1.29 b	22.8 ± 0.35 b	26.3 ± 0.65 c	1 ± 0.76	3.5 ± 0.76	3 ± 0.76
119.4	2616	*Cadophora melinii*	3.0 ± 0.00 a	–	37.0 ± 0.71 c	21.3 ± 1.59 b	33.4 ± 0.61 d	0 ± 1.59	2.5 ± 1.59	5.5 ± 1.59
120.5	2621	*Choniochaeta veluntina*	3.0 ± 0.58 a	43.7 ± 14.85 f	–	–	–	–	–	–
126.3	2617	*Cladosporium cladosporioides*	2.7 ± 0.33 a	–	45.7 ±1.70 e	42.6 ±0.33 e	40.1 ± 0.75 e	0 ± 1.32	4.5 ± 1.32	3 ± 1.32
125.1	2623	*Coniothyrium* sp.	–	–	47.0 ± 0.71 e	23.6 ± 1.32 b	20.2 ± 0.83 b	0 ± 1.32	4.5 ± 1.32	3 ± 1.32
122.4	2624	*Coprinellus* sp.	–	–	41.4 ± 0.64 d	38.9 ± 1.63 d	50.0± 0.78 f	0 ± 0.17	0.5 ± 0.17	0 ± 0.17
103.3	2625	*Cosmospora vilior*	2.3 ± 0.33 a	–	37.2 ± 0.88 c	38.1 ± 0.86 d	23.3 ± 0.77 c	0 ± 0.17	0.5 ± 0.17	0.5 ± 0.17
32.3	2534	*Cytospora chrysosperma*	5.0 ± 1.53 b	29.7 ± 4.91 d	–	–	–	–	–	–
118.3	2627	*Diaporthe eres*	10.7 ± 0.88 e	64.7 ± 17.75 h	–	–	–	–	–	–
*	2241	*Fusarium avenaceum*	7.0 ± 2.52 c	40.3 ± 12.60 e	–	–	–	–	–	–
*	2248	*Fusarium culmorum*	–	39.3 ± 15.72 e	–	–	–	–	–	–
*	2243	*Fusarium graminearum*	10.0 ± 1.16 e	55.3 ± 6.23 g	–	–	–	–	–	–
101.2	2649	*Glomerella cingulata*	15.3 ± 0.33 f	79.7 ± 10.37 i	–	–	–	–	–	–
101.4	2655	*Kabatiella microsticta*	2.3 ± 0.33 a	–	34.9 ± 0.42 c	47.1 ± 0.57 f	41.4 ± 1.52 e	0 ± 0.88	3 ± 0.88	1 ± 0.88
103.2	2661	*Lecythophora luteoviridis*	2.7 ± 0.33 a	–	60.4 ± 1.12 g	31.3 ± 0.50 c	33.3 ± 0.46 d	0.5 ± 0.76	3 ± 0.76	2.5 ± 0.76
25.6	2565	*Leucostoma niveum*	11.3 ± 1.76 e	27.3 ± 9.02 c	–	–	–	–	–	–
104.1	2677	*Phoma glomerata*	–	48.3 ± 1.67 g	50.9 ± 1.13 f	44.5 ±2.01 e	40.9 ± 1.28 e	0 ± 0.83	2.5 ± 0.83	0 ± 0.83
112.2	2674	*Phialocephala sp.*	–	–	37.4 ± 0.62 c	24.1 ± 0.48 b	25.4 ± 0.82 c	0 ± 0.17	0.5 ± 0.17	0 ± 0.17
111.3	2682	*Protoventuria alpina*	12.7 ± 1.20 e	41.7 ± 12.03 e	–	–	–	–	–	–
111.4	2683	*Rosellinia nectrioides*	–	–	22.2 ± 0.64 a	9.9 ± 0.61 a	8.3 ± 0.52 a	0 ± 0.88	3 ± 0.88	2 ± 0.88
12.7	2594	*Sclerotinia sp.*	14.0 ± 1.15 e	45.3 ± 16.27 e	–	–	–	–	–	–
120.4	2686	*Truncatella angustata*	19.0 ± 1.53 g	45.7 ± 4.63 g	48.7 ± 0.72 f	42.2 ± 0.40 e	49.9 ± 0.90 f	0 ± 0.00	0 ± 0.00	0 ± 0.00
15.10	2601	*Valsa salicina*	2.3 ± 0.33 a	44.7 ± 4.67 g	–	–	–	–	–	–
32.6	2604	*Valsella melostoma*	3.3 ± 0.88 a	29.0 ± 5.29 d	–	–	–	–	–	–

- F.g. - *F. gruminarum*, F.a.- *F. avenaceum*, and G.c.- *G. cingulata* in front of the potential antagonist isolates is also measured in dual-culture assay. Values are mean ± Standard error (SE) of three replicates. Treatments followed by the same letter are not significantly different from respective untreated control.

* Saskatchewan Microbial Collection Database, SMCD, Saskatoon, Sk.

In the greenhouse *in planta* assay, symptoms of infection by different fungi appeared after one week of inoculation, and the necrotic symptoms (anthracnose) were enlarged during the experiment. Symptoms ranged from small, discrete cankers to superficial necrosis (partial or total dark-brown discoloration) on bark and leaf (Figure 5-5). Plants treated with sterile water alone (control) showed no lesions. A significant difference was recorded between means of control and fungal treatments ($P < 0.05$). The disease severity ranged from a minimum at 13.7% ± 7.0 caused by *Botrytis byssoidea* to maximum at 79.7% ± 10.4 by *G. cingulata* in leaves. In addition, *Fusarium graminearum, Diaporthe eres, Phoma glumerata, Truncatella angustata* and *Sclerotinia* sp. also induced considerable lesions (Table 5-2 and Figure 5-6). Fungal species including *Leucostoma niveum, C. chrysosperma, Valsa salicina,* and *Valsella melostoma* caused severe open cankers on willow stems four weeks after inoculation.

The antagonistic relationship between potentially beneficial fungi and known phytopathogens was studied with a dual-culture assay in the three following groups; A= *F. graminearum,* B= *F. avenaceum,* and *C= G. cingulata*. Overall, there was no significant difference within variances of phytopathogen groups (one-way ANOVA, $P= 0.128$) (Figure 5-7). However, a significant difference between means of inhibition was recorded in each group (one sample t-Test, $P < 0.05$) (Table 5-2). The percentage inhibition (PI) was recorded as being highest for *Lecythophora luteoviridis* (60.4% ± 1.12) in group A. The inhibition zone (IZ) was highest (1 ± 0.76 mm) for *Cadophora luteo-olivacea* in this group (Figure 5-8, A). In group B, the highest IP was observed for *Kabatiella microsticta* (47.1% ± 0.57). *Cladosporium cladosporioides* had maximum IZ (4.5 ± 1.50 mm) compared to other isolates (Figure, 5-8 B). Among fungi co-cultured in group C, the IP was calculated as highest for *Coprinellus* sp. (50.0%± 0.78). Maximum IZ was recorded for *Cadophora melinii* (5.5 ± 1.59 mm) (Figure 5-8, C). *Rosellinia nectrioides* (strain CBS 449.89) had the least IP in all groups.

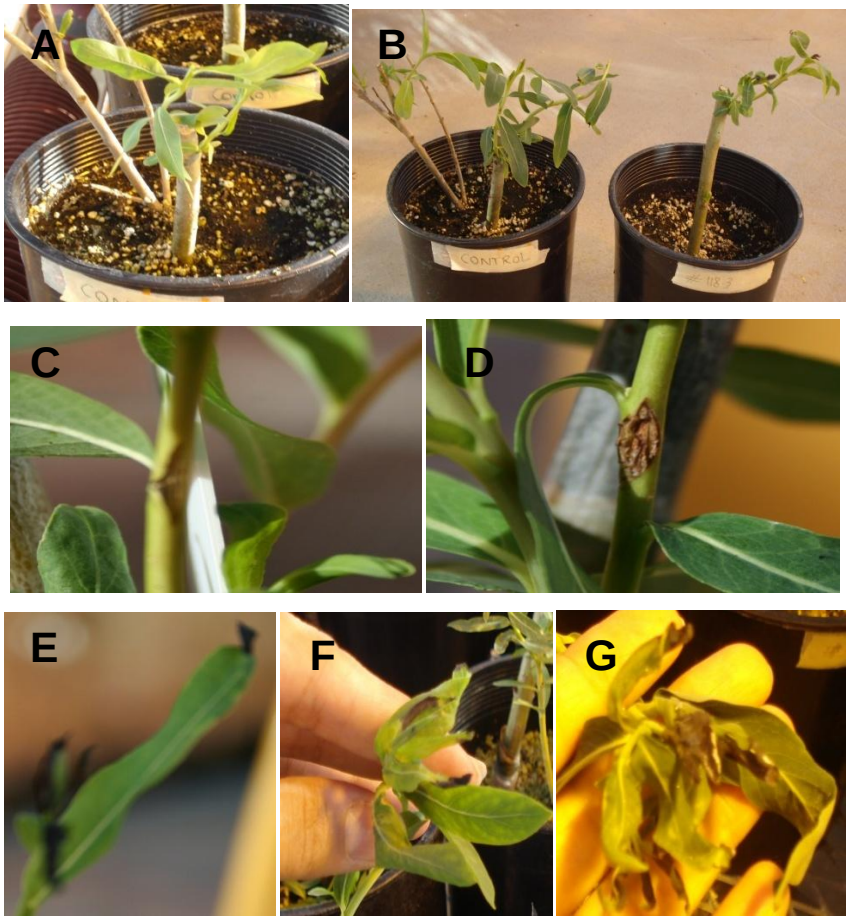


Figure 5-5. Symptoms of different fungal diseases on willow shoots and leaves two weeks after artificial fungal inoculation in greenhouse.

[Treatements: (A) Sterile water / control treatment on leaf, (B) Comparisson between sterile water inoculated (control) and phytopathogen inoculated willow cuttings, (C) Sterile water / control treatment on T-shap lesion on willow stem, (D) Open canker lesion on a young stem caused by *Valsa salicina*, (E) Leaf necrosis caused by *Glomerella cingulata*, (F) *Phoma glumerata*, and (G) *Protoventuria alpina*].

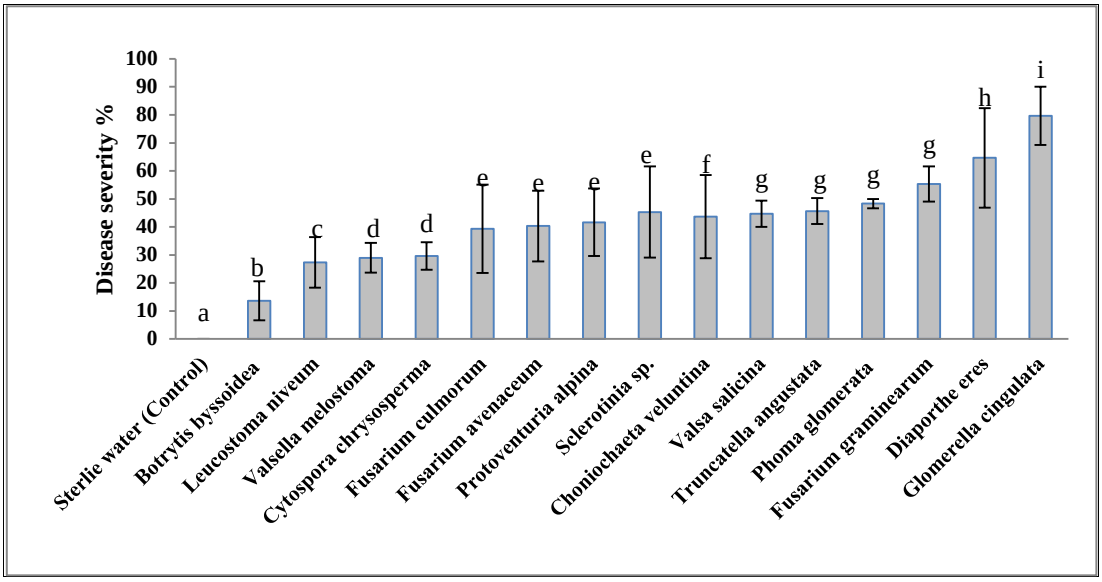


Figure 5-6. Comparison of disease severity based on leaf necrosis or bark canker lesions caused by potentially pathogenic fungi in greenhouse experiment. The data are the mean ± standard error of the infection percentage calculated repeated twice with three willow plants per each group. Treatments followed by the same letter are not significantly different from respective untreated control according to Kruskal- Wallis test at $P < 0.05$.

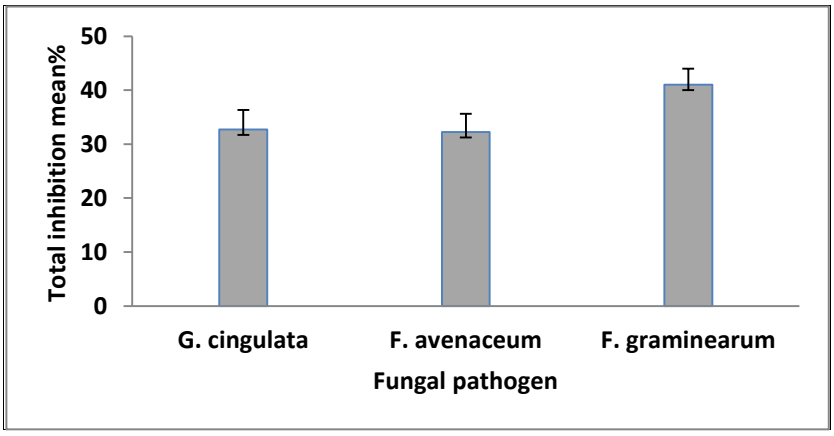


Figure 5-7. Average colony growth inhibition of fungal pathogens tested in dual-culture assay with fungal antagonists on PDA after 1-week of incubation. Values are mean ± standard error of inhibition percentages caused by potentially beneficial fungi.

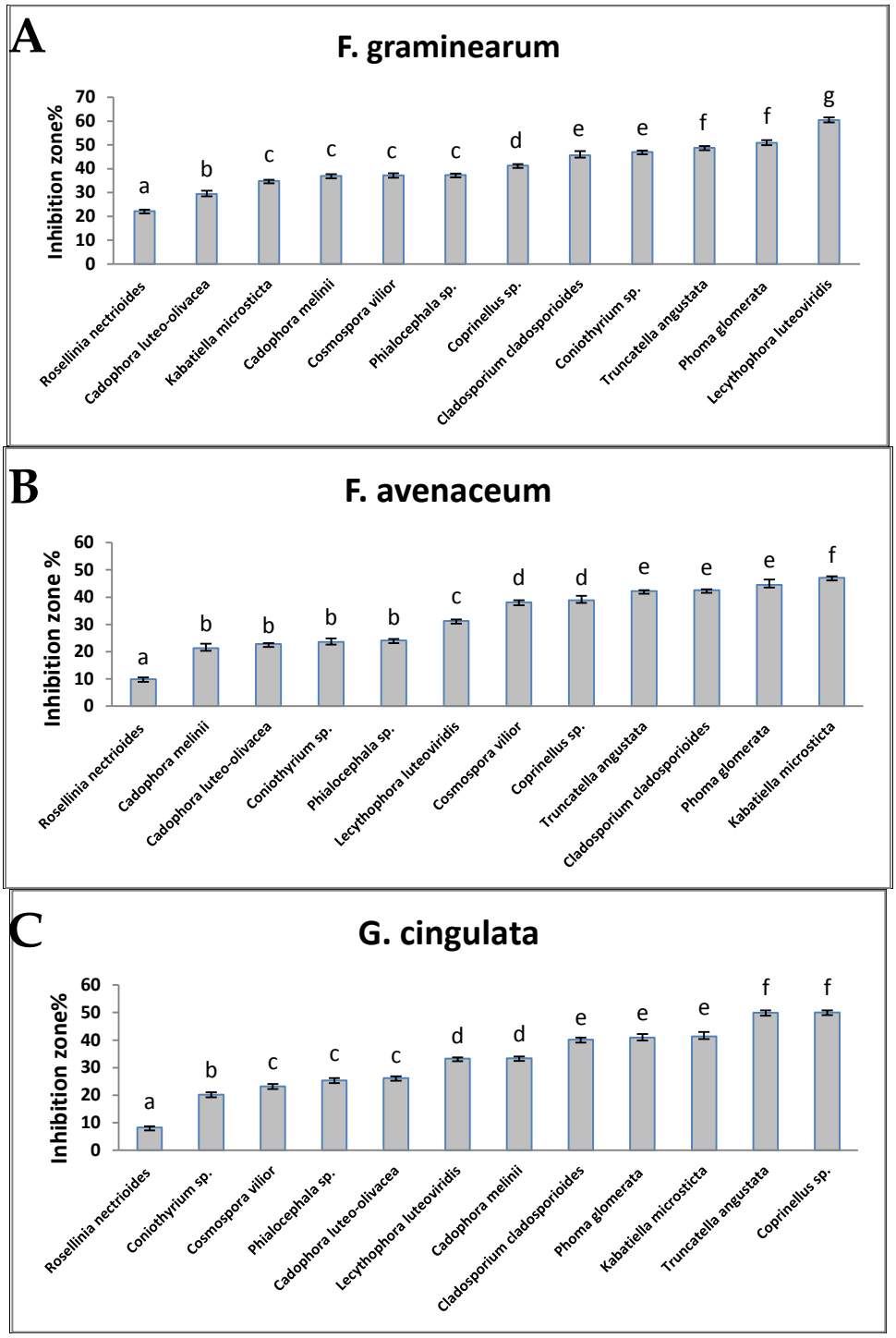


Figure 5-8. Comparison between colony growth inhibition of *F. graminearum* (A), and *F. avenaceum* (B) and *G. cingulata* (C) pathogens tested in dual-culture assay with fungal antagonists. Values are mean ± standard error (SE) of three samples in each treatment**.** Treatments followed by the same letter are not significantly different from respective untreated control according to Kruskal- Wallis test at $P < 0.05$.

5.6. Discussion

In this part of the study, we injected fungal isolates into wounded Golden Delicious apples and willow leaves to discriminate among the activities of fungal isolates in order to determine whether they were pathogenic or not. Fungal suspensions were gradually absorbed by tissues, and subsequently, phytopathogenic fungi caused brown patches on the apple or on leaves (leaf spot) as a necrosis symptom. Our results confirmed that the differences within control and non-symptomatic treatments were not significant ($P > 0.05$) while differences between control and symptomatic treatments were quite large, significant, and easily detected.

The pathogenicity of *G. cingulata, Diaporthe eres, Truncatella angustata, Sclerotinia* sp.*, Leucostoma niveum, C. chrysosperma, Protoventuria alpine* was confirmed in both apple and greenhouse experiments. The results showed that fungal isolates such as *G. cingulata, Diaporthe eres, F. graminearum,* and *Truncatella angustata* were obviously the most aggressive pathogens for willow leaves. According to the results, *G. cingulata* isolated from cuttings was identified in stem/leaf samples in our experiments as well. This fungus was previously reported to produce brown lesions on the surface of apples (Riordan et al., 2000) and is considered as the main causative agent for black canker of *Salix* spp. in Canada (Vujanovic and Labrecque, 2002). *G. cingulata* is reported as the causal agent of anthracnose leaf spot on maple species (LoBuglio and Pfister, 2008). *Diaporthe eres* is the causative agent of the most widespread disease in apple, known as European canker (Kosáry et al., 2008), and in our study, it was isolated from samples of cuttings. Our results also show the isolation of *Truncatella angustata* from cuttings, while its pathogenic effect has been proven in apple core rot (Eken et al., 2009). *Sclerotinia* sp., which is considered to be among the most important postharvest fungal pathogens in apples (Ramin et al., 2007) and an important cause of canola stem rot, was also separated from willow stem/bark and leaf samples. *Cytospora* and related teleomorphic species such as *Leucostoma* were found as canker producing agents in apple (Morrall. 2000). Cytospora canker is a fungal disease of many tree species in urban forests and orchards. This fungus is usually associated with open canker in wounded or stressed bark in hardwood plants such as in Canadian peach orchards (Buck et al., 1998). *C. chrysosperma* was isolated from aspen and cottonwood in the US in 2000 as pathogenic fungi in these tree species (Kepley and Jacobi, 2000). As this fungus was isolated from stem/leaf willow tissues in our study, the possibility of disease transfer from one tree species to other should be considered as a serious threat for distribution of fungal diseases in

willow plantations. The lesions caused by *F. avenaceum* and *F. graminearum* were observed on apple and in the greenhouse, although these fungi were not isolated from willow cuttings in our study.

The fungal species *Cosmospora vilior, Kabatiella microsticta, Cladosporium cladosporioides, Lecythophora luteoviridis, Cadophora melini,* and *Choniochaeta veluntina* (commonly isolated from willow cuttings) did not produce symptoms on Golden Delicious apples based on the indicated cut-off. This suggests that these fungi may be not considered as potentially pathogenic fungi for willow cuttings. However, *Valsella melostoma* and *Valsa salicina* (isolated rarely from willow stem/leaf) did not produce considerable lesions in apple; this may indicate that they are not natural pathogens for apple, or they could be facultative pathogens.

Inoculation of various fungi on apples is considered to be a feasible test for depicting and comparing phytopathogenicity of isolated fungi and to differentiate them from beneficial fungal species. To verify the pathogen status of the fungal pathogens, we re-isolated them from symptomatic apples, satisfying Koch's postulates. Since only some of the pathogenic fungal isolates were tested in the inoculation experiment in the greenhouse, and this selection may not have been representative of all existing pathogens in willow tissues, some potentially invasive fungal pathogens may have been overlooked in this test. In fact, bark is inhospitable for many fungal parasites, although the level of available nutrient materials, weather conditions and humidity can determine the invasion of pathogens through cracks or fissures. Furthermore, the existence of lenticels in older bark can provide for and support larger fungal populations compared to smooth bark surfaces (Buck et al., 1998).

In dual-culture assays, fungal isolates responded differently to fungal pathogens. Among potentially antagonist fungi, *Rosellinia nectrioides* showed weakest growth inhibition against all three groups of pathogens. On the other hand, *Cladosporium cladosporioides, Truncatella angustata, Phoma glomerata,* and *Coprinellus* sp. depicted strong growth inhibition abilities against all pathogens (PI>35 %). However, *Lecythophora luteoviridis, Coniothyrium* sp. (ICMP 17485), and *Phialocephala* sp. (isolate L48) depicted noticeable growth inhibition only when co-cultured with *F. graminearum.* The same was true for *Cosmospora vilior* when co-cultured with *F. avenaceum* and *F. graminearum* but not for *G. cingulata*. *Cadophora luteo-olivacea* also experienced moderate growth inhibition in all dual-culture groups. Comparison of the means of inhibition percentages of the three indicated fungal phytopathogens tested in dual

culture experiments demonstrated that *F. graminearum* experienced the highest percentage of inhibition (Figure 5-7). However, it was not significantly different from other pathogens ($P>0.05$). *F.avenaceum* and *G. cingulata* experienced relatively equal growth inhibition but less than *F. graminearum*. In addition, *F. graminearum* strain was not inhibited by fungi such as *Phoma glomerata. Kabatiella microsticta* affected mostly the *F. avenaceum* growth (Figure 5-8, B). Interestingly, pathogenic *Truncatela angustata* consistently antagonized all pathogens tested in dual-culture (*G. cingulata, F. gruminarum* and *F. avenaceum*) probably through the competition.

In this regard, there are many factors which play important roles in determining the rate of inhibition. Interference and competition among fungal organisms involves several factors such as behavioral or chemical mechanisms which can affect and influence the presence and growth of the competitor (Mille-Lindblom and Fischer, 2006). The fungal inhibition behavior is reciprocally related to the increase in weight of mycelia and production of extracellular enzymes by the antagonist fungus in dual culture assays (Radjacommare et al., 2010). Moreover, fungal antagonism is determined as a mechanism which can prevent the invasion of other fungi (Jeffries, 1995). On the other hand, fungi can overgrow in the presence of each other without high inhibitory effects on each other. Certainly, the evolutionary commensalism depends on the environmental conditions and nutrient material availability as well as presence or absence of other fungal micro- organisms (He et al., 2006). In our experiment, creating 1.5 mm-wide mycelial zones formed by fungi such as *Truncatella angustata*, *Phoma glomerata*, *Kabatiella* sp., *Lecythophora* sp. and *Coprinellus* sp., it was observed that hyphal interference existed between these fungi and pathogenic (*F. gruminarum, F. avenaceum,* and *G. cingulata*) isolates. However, there are very limited studies concerning the rate of fungal pathogen inhibition by interference competition. In this experiment, the interference interactions supports the hypothesis of competition or co-existence of these fungal isolates, although the fungus-fungus interactions may considerably differ in natural environments compared to what was observed during the dual-culture experiments (He et al., 2006).

In conclusion, the study revealed antagonistic activity of some of the isolated fungi, found on willow phyllosphare, against the known pathogenic fungi mentioned earlier. These data clearly indicate that it is important to recognize the beneficial fungal species in willow trees and investigate their interactions with the pathogenic species in order to build management strategies to enhance healthy plantations. Further research is needed to better clarify the fungal antagonistic

relationships. For this reason, we believe that isolation and characterization of both pathogenic and biocontrol fungal communities associated with willow plantations could be the first step in answering the above challenges. The dual-culture assays presented here provided interesting results for evaluating isolated fungal communities on willow phyllosphare. Furthermore, this can be followed by fungus-fungus greenhouse and field-based experiments. However, evaluation and measurement of the results can be better guaranteed *in vitro* because of the relatively uniform conditions.

6. GENERAL CONCLUSION

Our results support the finding that major pathogenic fungal isolates may have originated from willow-cuttings or from previous land crops. In this study, several fungal taxa that were isolated from willow above-ground tissue are still not precisely identified and need further investigation. Important consideration should also be given to the cultivar choice for proceeding with SRIC (Short Rotation Intensive Cultures). If we select a resistant willow cultivar in combination with other management practices we can eliminate the opportunity of pathogen fungi and increase the manifestation of beneficial fungi. Biological controls have the potential to suppress pathogens in this way. We believe that these results can improve the management strategy of willow SRIC. Although these results appear to be successful, it has to be kept in mind that they trust in the taxa abundance observation which was highly dependent on the abundance of sequences represented in the BLAST database. This situation can be generated because of the biases in GenBank sequences (Bidartondo et al., 2008). Because newly identified sequences are always increasing, it is necessary to find a more practical and accurate fungal database.

Non-indigenous plant pathogenic fungi can have huge effects on agriculture and agro-forestry systems. Prevention of not-yet-discovered (exotic) fungal pathogens also pose some challenges. Most of the functional fungal groups related to willow plants are yet unknown, especially those classified as the *Ascomycota* phylum, which can be significant competitors of pathogenic fungi. Some pathogenic fungi often escape a visual inspection, since do not produce disease symptoms in a part of their lifecycle, so detection of their presence is difficult. The best way to prevent introduction of pathogenic strains is to isolate or asses them using specific molecular primers before entering the farmlands. The present study provides new insights into the genetic connectedness among different fungal species associated with willow cuttings. Also,

the techniques used in this study can be considered as a reliable and effective procedure for more epidemiological studies related to the health status of cuttings. It also assists in the importance of monitoring imported willow cutting fungal community structures in order to prevent introducing exotic fungal pathogens. In addition, this study supplies new data on fungal biodiversity in Canadian willow plantations. To our knowledge, this is the first use of the PCR-DGGE method to evaluate the fungal community composition in willow cuttings. With the help of this molecular method we could pass over the cultural-phylogenetical identification and detect other fungal taxa that are not isolated using conventional laboratory methods.

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