The Isolation, Identification and Characterization of Endophytes of Switchgrass (*Panicum virgatum* L.), a Bioenergy Crop

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ABSTRACT

It has been established that perennial grasses harbour different types of endophytic bacteria and fungi. Switchgrass (*Panicum vergatum* L.) is identified as a model perennial energy crop. This study was conducted to explore fungal and bacterial endophyte communities inhabiting switchgrass cultivars of Quebec. The primary focus of this study was to isolate the endophytes, and provide taxonomic identifications based on comparative analysis of ITS rDNA gene sequences. A total of 145 endophytes isolates were recovered (52 bacteria and 93 fungi) from whole plant samples collected at early vegetative, and full reproductive stages. Five and nine different taxa of bacteria and fungi were identified, respectively. We evaluated the antagonistic activity of some endophytes against several fungal pathogens and selected candidate endophytes for future introduction into commercial switchgrass cultivars for biomass enhancement. We demonstrate the vertical transmission ability of some endophyte from one switchgrass generation to the next using species-specific primers. Artificial inoculation of young switchgrass seedlings with selected bacterial endophytes hold promise as a method of reinfection switchgrass seedlings.
Résumé

Le panic érigé (*Panicum vertatum* L.) est reconnu comme une des plantes modèles pour la production de biomasse végétale. Il est connu que la plupart des plantes vasculaires étudiées à ce jour sont colonisées par des champignons et bactéries endophyte. Cette étude avait pour but d’explorer les communautés d’endophytes présentes dans différents cultivars de panic érigé au Québec, pour ensuite isoler les endophytes et effectuer leur identification taxonomique en comparant leur séquence ITS rADN. Nous avons obtenu un total de 145 isolats (52 bactéries et 93 champignons) venant de feuille de plante au stade végétative et au stade reproductive.

Une fois les isolats identifiés, nous avons obtenu cinq différents groupes taxonomiques pour les bactéries et neuf pour les champignons. Nous avons évalué le potentiel antifongique des différents endophytes bactériens, avec pour objectif d’identifier les candidats potentiels à la ré-inoculation de cultivar de panic érigé commercial afin augmenter leur production de biomasse. À l’aide de séquences d’amorces spécifiques, nous avons pu démontrer la transmission verticale des endophytes.
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Chapter I Introduction

1.1 Problem Definition

The need to find sustainable alternatives to replace increasingly expensive fossil fuels and to reduce greenhouse gas (GHG) emissions has peaked interest in biofuels around the world. Biofuel produced from renewable biological resources such as plant biomass and treated municipal and industrial wastes, is envisaged as being part of the solution. For example, the Québec Energy Strategy for 2006-2015 was established in order to reduce the consumption of fossil fuels and promote renewable fuels. In particular, the use of annual grains such as corn for biofuel production is discouraged in Québec and priority is given to energy crops. Perennial energy crops more effectively capture and store solar energy as biomass through photosynthesis, increase the overall energy produced per hectar, and are productive on marginal farmland. In 1985, the USA began a 5-year program to develop herbaceous energy crops. Switchgrass \((Panicum \textit{vergatum} \textit{L.})\) was identified as a model herbaceous energy crop species (Samson, 1991). Switchgrass had a number of positive attributes especially being a productive long-lived perennial crop with high resource use efficiency and good adaptability to marginal soils (McLaughlin and Kszos, 2005). The first scientific studies in Canada on switchgrass were subsequently conducted by Resource Efficient Agricultural Production (REAP)-Canada in 1991. These studies proved that switchgrass is well adapted to Québec and Ontario environment and productive in Quebec
and Ontario (Samson, 1997), and that it has an excellent energy balance, low cost of production and an ability to increase landscape carbon sequestration and an ability to increase landscape carbon (Donner and Kucharik, 2008). Because of the above-mentioned attributes, more efforts focused on switchgrass development as an energy crop could benefit Quebec's agriculture, and represent a new source of income for Quebec farmers. Already, 116 Quebec farms dedicate 816 hectares to switchgrass production, a 50% increase compared to what was planted in 2008 (REAP-Canada, unpublished). The development of a national capacity to utilize perennial herbaceous crops, including the native prairie plant switchgrass, as biofuels could benefit Quebec's agriculture by optimizing productivity on marginal farmland or degraded lands and by providing a new source of income for Quebec farmers.

1.2 Rational for research

New approaches are required to assess and improve the genetics and cultural management of the crop for Quebec's marginal farming areas. For example, perennial grasses are the hosts of fungal and bacterial endophytes that systemically colonize the roots or the above ground portions of grasses and their seeds (Ryan et al., 2008; Schulz and Boyle, 2005). The close link between endophyte fitness and its host grass is presumed to align the interests of both partners towards a mutually beneficial cooperation. The endophytes gain shelter, nutrition, and dissemination via host propagules, and can
contribute an array of host fitness enhancements including improvement of vigor, leading to superior agronomic qualities, greater drought tolerance, and increased resistance to herbivory and also against pathogen. (Clay, 1990; Lodewyckx et al., 2002; Schulz et al., 1993; Schulz et al., 2002). However, the importance and use of endophytes in switchgrass remains poorly understood.

1.3 Hypotheses

The objectives are supported by three hypotheses:

I. Endogenous endophytes of switchgrass can be successfully isolated on synthetic microbiological media.

II. Endogenous endophytes can be detected in plantae using polymerase chain reaction (PCR) method.

III. Switchgrass endophytes are transmitted from maternal plant to offspring via seeds.

1.4 Objectives

The objectives were:

Objective 1: To study the distribution and frequency of fungal and bacterial endophytes in switchgrass cultivars established in field sites across in southwestern region of Quebec, followed by their isolation and characterization using accurate and sensitive detection methods.
Objective 2: To establish that endophytes are inherited by host offspring in a vertical transmission mode via seeds.

Objective 3: To study the biological activity of selective isolated endophytes.
Chapter II Literature Review

2.1 Switchgrass

2.1.1 Switchgrass as biofuels feedstock

Switchgrass (Panicum vergatum L.) is a perennial warm-season (C₄) grass native to the grassland of North America (Lemus et al., 2002). Switchgrass has a wide native geographic range and has evolved into two ecotypes: Lowland ecotypes are tetraploid, are characterized as vigorous, tall, thick-stemmed and is adapted to wet conditions. The upland ecotypes are hexaploid or octaploid (Lemus et al., 2002), are characterized as short, rhizomatous and thin-stemmed, and are adapted to drier conditions. Both ecotypes of switchgrass offer a significant opportunity to improve agricultural sustainability as they decrease erosion and improve water quality, when compared to row crops. Highly eroded land will benefit the most from the perennial nature of switchgrass.

The selection of switchgrass as a biofuel feedstock is based on several significant attributes, including its efficiency in mitigating greenhouse gases (GHG) emissions by sequestering large amounts of carbon in its extensive root system (Donner and Kucharik, 2008), its high biomass mainly having a high concentration of lignin and cellulose, and low
amounts of water, nitrogen and ash (Lemus et al., 2002; Samson, 1997; Samson, 1991). Additionally, it has high productivity across a wide geographical range, superior suitability for marginal quantity land, low water and nutrient requirements and positive environmental attributes. Because of these attributes, research projects across North America and Europe were initiated to pinpoint important new information on the yield potential of this species as a biofuel crop. Research focused on evaluation of the most promising existing varieties in regional field trials, cultural treatments to enhance the establishment and growth of stands, breeding for superior yield, identification of physiological markers for assessing and promoting improved growth and development, and the development of tissue culture techniques for biotechnological improvement (Berdahl et al., 2005; Casler and Boe, 2003; Cassida et al., 2005; Elbersen et al., 2001b; Lee and Boe, 2005; Madakadze et al., 1999; Muir et al., 2001; Sanderson et al., 1999; Vogel et al., 2002). These tasks combined the interrelated needs of realizing maximum near-term requirements in productivity potential through cultural improvement and the longer-term requirements for enhancing and protecting yield capacity.

In the USA, development in switchgrass is mostly aimed for cellulosic ethanol production (Bouton 2008) in order to displace fossil fuels, thereby, reducing expenditure on imported fuels. While in Quebec and Ontario, the focus has been more on agri-fiber pellet that is used in commercial boilers such as in greenhouse industries (Samson and
It is estimated that these two provinces could produce 14 millions tons of switchgrass biomass if 20% of crop land and 40% of forage land would be converted to switchgrass production (Samson and Stamler, 2007). This technology has the potential to produce 770–890% more net energy gain/ha than growing grain corn for ethanol. However, it remains substantially less efficient than direct combustion of energy grasses or corn silage biogass as means to produce energy from farmland.

2.1.2 Switchgrass Cultivars used in North-Eastern States, Québec and Ontario

There are currently 20 different switchgrass cultivars available on the market. A great deal of effort is focused presently on breeding and creating new lines (Lemus et al., 2002). The most widely used cultivars in North-Eastern America and recommended by experts is Cave-in-Rock (Lemus et al., 2002). Others such as Sunburst, Shelter and Forestburg are also suitable cultivars for production in northern locations (Samson, 2007).

Warm-season grasses such as switchgrass are increasingly being cultivated in North America for summer forage and biomass production. The cooler temperatures and shorter growing seasons typical of Canadian production areas, are major limiting factors to warm-season grass production in these areas. Assessment of the morphological development and relationship of growing degree-days (GDD) to plant morphology and tiller characteristics were evaluated in nine cultivars of switchgrass. Cave-in-rock switchgrass, a cultivar
originating from Southern Illinois (Elbersen et al., 2001a) showed increases in tiller number and had the highest ground cover ratings after a three-year trial conducted in southwestern regions of Quebec, thereby demonstrating that it is well adapted to Quebec climate and soil type (Madakadze et al., 1999). The cultivar Blue Jacket is an upland ecotype evolved from Sunburst, a cultivar originated in South Nebraska, and its main advantage over other cultivars is its superior seedling vigor due to heavy seed set. This cultivar is well adapted to sandy soils and low rainfall than Cave-in-Rock (Boe and Ross, 1998). Tecumseh switchgrass, an upland ecotype evolved from Summer which originated from South Dakota, well adapted to heavy soils, and requires a lot of precipitation (Elbersen et al., 2001a; Samsons, 2011). Finally, the cultivar Sand Lover is a population of switchgrass derived from cultivar NU 94-2. It is an upland ecotype. This cultivar originated in Oklahoma and is well adapted to sandy soils and dry environments (Taliaferro, 2002).

2.2 Endophytes of grasses

For the purpose of this study, the term endophytes will only refer to bacteria or fungi that establish a mutualism or commensalism interaction with the aerial parts of grasses. Endophytes are present in the spaces that run parallel to the long main axis of leaves and stem (Clay, 1990). These organisms have the ability to absorb freely available nutrients found in the intercellular spaces or obtain their nutrients from the surrounding cells (Schulz and Boyle, 2005; Schulz et al., 1999).
Endophytes are microorganisms that inhabit healthy plant tissues during at least one stage of a grass life cycle, and do not cause any apparent symptoms of disease or negative effects. Presently, multiple studies have shown that several plant species are hosts to a great deal of endophytic biodiversity. According to Schulz and Boyle (2005), some endophytic species can occur in unique ecological niches and may be sources for a variety of bioactive metabolites that may have great potential for pharmacological applications.

Many fungal endophytes are seed-borne and migrate in the seed during seed germination. On plants that propagate vegetatively, the endophytes use the reproductive tissues as vector for the infection in new plants. In order to colonize non-infected plants, the fungi gain access to host cells using infective specialized structures such as appressoria and haustoria that develop intracellularly. They can also penetrate directly through the cell wall or enter through the stomata and the substomatal chambers (Schulz and Boyle, 2005).

Grasses and fungi have a long history of symbiosis ranging in a continuum from mutualisms to antagonisms (Schulz and Boyle, 2005). This continuum is particularly evident among symbioses involving the fungal genus *Epichloë*. In the more mutualistic symbiota, the *Epichloë* endophytes are vertically transmitted via host seeds, and in the more antagonistic symbiota they spread and suppress host seed set (Schardl et al., 2004). Generally, the endophytes gain shelter, nutrition, and dissemination via host propagules,
and can contribute to host fitness enhancements including protection against insect and vertebrate herbivores, enhancements against drought tolerance and nutrient status, and improved growth particularly of the roots. Recent advances in endophytes’ molecular biology promise to shed light on the mechanisms of the symbioses and host benefits (Malinowski and Belesky, 2000; Pavlo et al., 2011). For example, Pavlo and coworkers (2010) applied enzymatic activity and Real-Time PCR assays, to shed light on some important plant mechanisms (e.g., ISR and SAR) for protection against pathogen and are up-regulated by the presence of endophyte (see section 3.5). Fungal endophytes can be classified into three different ecological groups: the mycorrhizal fungi, the balansiaceous and the non-balsaniaceous taxa. Mycorrhizal associations will not be covered, as they are not the subjects of this research.

2.2.1 Balansiaceous fungal endophytes

The balansiaceous or ‘grass endophytes’ is a group of closely related fungi with specific ecological requirements and adaptations that are different from other fungal endophytes. The balansiaceous endophytes Epichloë and Balansa, (Anamorphs: Neotyphodium and Ephelis, respectively) belong to the Ascomycetes. They are the most studied above ground endophytic interactions because of their ecological and economical impact. They grow systemically and intercellularly within all above ground plant organs of grasses, rushes and sedges (Schulz and Boyle, 2005). They are dependent on nutrients
present in the apoplast for growth. A signal communicating system between the host and the plant insures a perfect balance between the host and the endophyte virulence (Schulz and Boyle, 2005) with benefits to both partners: the endophyte gains access to nutrients and protection from abiotic stress, and the host becomes more resistant to insect or pathogen attack due to the presence of alkaloids or the production of chitinase enzymes (Schulz and Boyle, 2005). Many fungal endophytes are seed-borne and migrate in the seed during seed germination processes.

### 2.2.2 Non-Balansiaceous fungal endophytes

In contrast to balansiaceous endophytes, the non-balansiaceous endophytes are a diverse group of fungi both from the taxonomic and life-history strategy point of view. These fungi are Ascomycetes and can belong to any of the following genera: *Acremonium, Alternaria, Cladosporium, Conithryium, Epicoccum, Fusarium, Geniculosporium, Phoma, Pleospora, Colletotrichum, Guignardia, Phyllosticta, Pestalotiopsi, and Lophodermium*. They are found in all the organs and have been shown to be present in all sampled plants (Schulz and Boyle, 2005). Many of these fungi produce an array of secondary metabolites (for review Schultz et al. 2002) with varied biological activity (refer to section 4; Bashyal et al., 2006; Suryanarayanan et al., 2009b).

The colonization of non-balansiaceous endophytes can be localized or systemic. They can grow intercellularly or intracellularly and form different types of interactions
varying from non-aggressive, mutualistic to pathogenic. The non-balansiacous endophyte composition population of a plant varies according to the tissues (root, stem, leaf, flower). The fungi are not obligate host specific; they have a certain level of adaptation to different hosts. While others are more specific and can only be found in specific organs of specific plant (Schulz and Boyle, 2005).

2.3 Bacterial endophytes

Endophytic bacteria have been found in virtually every plant studied, where they colonize the internal of their host plant and can form different relationships including symbiotic, commensalistic, mutualistic and trophobiotic (Ryan et al., 2008). Most endophytes appear to originate from the rhizosphere or the phyllosphere, however some may be transmitted via seeds. They are able to colonize the internal tissue of the plant showing no external sign of infection or negative effect on their host (Schulz and Boyle, 2005). They are often isolated from surface-sterilized tissues or extracted from internal plant parts. Both gram-positive and gram-negative bacterial endophytes have been isolated from several tissue types in numerous plant species. However, several different endophytic bacteria may reside within a single plant (Kobayashi and Palumbo, 2000). These endophytes either remain localized at their entry points or spread to other parts of the plant (Hallmann et al., 1997).
Bacterial endophytes cover a significant range of Gram-positive and Gram-negative bacteria (Lodewyckx et al., 2002). Most bacterial endophytes belong to *Cellulomonas, Clavibacter, Curtobacterium, Bacillus, Sphingomonas, Pantoea, Pseudomonas* and *Microbacterium* genera (Kang et al., 2007; Ryan et al., 2008; Thomas et al., 2007). They will colonize the host without external signs of infection or negative effects on the host (Rosenblueth and Martinez-Romero, 2006). Bacteria from the same genera can colonize both the roots and the aerial parts depending on the specificity of the host and the bacteria (Lodewyckx et al., 2002). Many bacterial endophytes are seed borne. On plants that propagate vegetatively, the endophyte can use the reproductive tissues as vector for infecting the new plant. In order to colonize non-infected plant, bacterial endophytes secrete cellulolytic, pectinolytic, cell wall degrading (endoglucanase and polygalacturonase) enzymes. These enzymes are useful to penetrate the host (Rosenblueth and Martinez-Romero, 2006).

As with their fungal counterparts, bacterial endophytes also produce secondary metabolites exhibiting antifungal and antibacterial properties. For example, fengycin, bacillomycin D, Zwittermicin A, Iturin A, Surfactin are peptides with strong antimicrobial activities and are produced by *Bacillus spp* (Athukorala et al., 2009; Ramarathnam et al., 2007).
Only few plants have ever been completely studied relative to their endophytic biology. Consequently, the opportunity to find new and beneficial endophytic microorganisms among the diversity of plants indifferent ecosystems is considerable.

2.4 Beneficial roles of endophytes

Generally, endophytic bacteria and fungi can promote plant growth and yield and can act as biocontrol agents. Endophytes can also be beneficial to their host by producing a range of natural products that could be harnessed for potential use in medicine, agriculture or industry (Ryan et al., 2008; Schulz and Boyle, 2005; Schulz et al., 2002; Schulz et al., 1999). In addition, it has been shown that they have the potential to remove soil contaminants by enhancing phytoremediation and may play a role in soil fertility through phosphate solubilization and nitrogen fixation (Ryan et al., 2008). There is increasing interest in developing the potential biotechnological applications of endophytes for improving phytoremediation and the sustainable production of nonfood crops for biomass and biofuel production.

2.4.1 Effects on Plant Physiology

Endophytes are known to have many effects on their hosts. Infected plants may produce more inflorescences and seeds than uninfected plants. This can be explained by a
greater vegetative vigor of infected plants (Clay, 1990). Seeds of some grass species (e.g., tall fescue and perennial ryegrass) germinate more rapidly and grow faster when infected by an endophyte. Thus, resulting in faster growth of the infected seedling. Fungal endophyte-infected seeds contain higher concentrations of alkaloids, which may explain this faster germination/growth rate. The alkaloids concentration makes it less likely for the seed to be eaten by vertebrates and invertebrates plant and seed feeders (Clay, 1988). Grasses infected by endophytes have tillers that are more profuse and spread horizontally via stolons and rhizomes (Clay, 1990). Enhanced growth rate of infected plants was also demonstrated with perennial ryegrass and purple nutsedge (Latch and Christensen, 1985).

### 2.4.2 Photosynthesis

Endophytes influence the source and sink mechanism of the plant. Experiments conducted on photosynthate metabolism reveal that endophytic fungi were constantly transforming plant sucrose into sugar alcohols that are unavailable to the plant to metabolize (Smith et al., 1985). If the level of photosynthetic activity of the plant is high, the plant metabolism in charge of processing the outcome of the photosynthesis (sugar) cannot work fast enough. Thus, there is a sucrose build up, which in turn will trigger a plant feedback inhibition mechanism, leading to reduction in photosynthetic activity. Therefore, entophytic fungi prevent the feedback inhibition of photosynthetic rates, allowing higher photosynthetic rates and subsequently increase the average growth of the plant. In the case
of root-borne endophytes, siderophores are produced that can increase the solubility of nutrient in the rhizosphere (Rosenblueth and Martinez-Romero, 2006).

2.4.2 Drought resistance

Drought stress usually induces a series of adaptations in the plant, in order for it to survive. These adaptations include mechanisms of drought avoidance, tolerance, and recovery from drought. Endophytic presence can impact these different adaptations.

Drought avoidance is the series of mechanisms by which the plant attempts to maintain an efficient water supply to above ground organs or conserving water during periods of soil water deficit. A good way to improve and maximize water uptake is by developing a deeper and denser root system. Perennial ryegrass, tall fescue, meadow fescue infected by fungal endophytes showed an increase in root dry matter, root hair length, and decrease root diameter (Malinowski and Belesky, 2000; Thomas et al., 2007). These traits increase root surface, leading to increase in water and minerals absorption. For example, Papaya infected by the bacterial endophytes Pantoea ananatis, Bacillus subtilis and Microbacterium esteraromaticum showed an increase in root development (Kang et al., 2007; Thomas et al., 2007). The superior root system is a simple way to maximize the uptake of available water in the soil, thus improving the drought capacity of the plant.
Another drought avoidance technique is to reduce transpiration. The stomata of Tall fescue and meadow fescue endophyte-infected plant close faster under water stress conditions than non-infected plants (Malinowski et al., 1999; Turner, 1986). The internal plant hormones balance has to be modified in order to influence the stress behaviour of the plant. Of interest is the presence of auxin, a major plant hormone that is involved in the drought resistance signalling pathway (Chaves et al., 2009). Clay (1990) found that auxin can be produced by the endophyte Balansia epichloe. Water content of tiller bases in endophyte-infected plants are maintained at higher levels than those in non-infected plants during drought conditions. This may be due to a greater accumulation of solutes in endophyte-infected tissues.

Drought tolerance is series of adaptations that enable the plant to withstand water deficits. A good way to cope with drought is to have carbohydrate reserves used up during stress periods. An important part of drought tolerance adaptation is to be able to maintain normal osmotic pressure. Accumulation of solutes in tissue helps maintain the turgor pressure, which in turn facilitates physiological and biochemical processes. Endophyte-infected plants produce a number of these solutes; water-soluble sugars, mannitol and arabitols, proline and loline (Malinowski and Belesky, 2000). Endophyte-infected plants
help maintaining cell wall elasticity in the same way as they do maintain osmotic pressure, which further help the biological processes.

The combination of drought avoidance and tolerance mechanisms determines the natural potential of a plant to withstand drought. Endophytes can play a key role on many of the different strategy used, thus increasing the drought tolerance of plant.

2.4.3 Resistance to herbivory

Endophytes are known to increase resistance to herbivory (Clay, 1990). Several findings showed that endophyte-infected plant materials reduce feeding and egg laying intensity of aphids, large milkweed bugs, and fall armyworms (Clay, 1988). The endophyte-infected plant reduces the survival, growth and developmental rates of feeding insects. These effects, are due to the presence of secondary metabolites, with insecticidal property, produced in plant tissues by the endophytes (Johnson et al., 1985; Suryanarayanan et al., 2009a; Suryanarayanan et al., 2009b). These secondary metabolites are often alkaloids in nature. To name a few: the Ergots alkaloids like clavines, lysergic acid, ergopeptides are the cause of fescues toxicosis in grazing livestock (Malinowski and Belesky, 2000). Peramine is another alkaloid that is found on endophyte-infected grasses; it has insect-feeding deterrent properties, but in this cases does not impact mammalian herbivory (Malinowski and Belesky, 2000). Endophyte-infected grasses have been reported to be also more resistant to soil-born nematodes, with the resistance probably attributable to alkaloids
present in roots or secretion of phenolic-like compounds into the rhizosphere (Malinowski and Belesky, 2000).

2.4.4 Reduction in disease occurrence

Numerous reports have shown that endophytic microorganisms can have the capacity to control plant pathogens (Krishnamurthy and Gnanamanickam, 1997; Rodriguez Estrada et al., 2011), insects (Azevedo et al., 2000) and nematodes (Hallmann et al., 1997). In some cases, they can also accelerate seedling emergence, promote plant establishment under adverse conditions (Chanway, 1997) and enhance plant growth (Bent and Chanway, 1998).

It is believed that certain endophytic bacteria trigger a phenomenon known as induced systemic resistance (ISR), which is phenotypically similar to systemic-acquired resistance (SAR) (van Loon et al., 1998). ISR is effective against different types of pathogens but differs from SAR in that the inducing bacterium does not cause visible symptoms on the host plant (van Loon et al., 1998). Bacterial endophytes and their role in ISR have been reviewed recently by Kloeper & Ryu (2006). Pavlo et al. (2010) demonstrated that the bacterial endophyte of potato, *Pseudomonas* spp. increased resistance toward the pathogen *Pectobacterium atrosepticum* by priming the host's defense responses. With the help of enzymatic activity assay and Real-Time PCR, they discovered that the plant antioxidant
system that produces superoxide dismutase (SOD), catalase, guaiacol peroxidase (GPOX) and ascorbate peroxidase (APX) was moderately activated as well as both the ISR and the SAR pathway by the presence of endophyte. Reduced nematode populations were associated with endophyte infection in tall fescue, both in pot culture and in field trials (Pedersen et al., 1988; West et al., 1988). Possible mechanisms by which endophytes could inhibit plant pathogens include competition for resources (Sturz et al., 2000), induction of generalized defense responses, and the production of antimicrobial compounds (Athukorala et al., 2009; Ramarathnam et al., 2007).

2.5 Secondary metabolites

Endophytes have been shown to prevent disease development through endophyte-mediated de novo synthesis of novel compounds and antifungal metabolites. Investigation of the biodiversity of endophytic strains for novel metabolites may identify new drugs for effective treatment of diseases in humans, plants and animals (Strobel et al., 2004).

Most of the effects of fungal endophytes on their hosts can be associated with the production of secondary metabolites, more specifically alkaloids, that are generally host-specific and can be extracted from endophyte cultures outside the host (Clay, 1990; Schulz and Boyle, 2005; Suryanarayanan et al., 2009a; Suryanarayanan et al., 2009b). Alkaloids are defined as naturally occurring molecules containing nitrogen atoms (Clay, 1990). It has
been shown that greater resistance to pathogens and predators is usually associated with antimicrobial metabolites (Schulz and Boyle, 2005). These alkaloids become part of the plant tissue and are in part responsible for the poisonings of grazer animals and insect herbivore. Endophyte-infected grasses contain a series of alkaloids not found in uninfected grasses.

Generally, metabolites produced by endophytes can be isolated from pure culture of endophytes. The balansiaaceous endophytes produce diverse array of secondary metabolite, including peramine and lolines and the anti-vertebrate alkaloids lolitrem B and ergovaline (Schulz and Boyle, 2005) that are harmful for mammals. While the non-balansiaaceous endophytes are known to produce a diversity of metabolites that are known to have herbicidal (e.g., brefeldin A produced by \textit{Aspergillus clavatus}), anti-bacterial (e.g., pyrrocidine A and B produced by \textit{Acremonium zeae}), anti-viral (e.g., mellein produced by \textit{Penicillium janzcewskii}), anti-fungal (e.g., pyrrocidine A and B produced by \textit{Acremonium zeae}), and anti-cancer (e.g., vincristine produced by \textit{Fusarium oxysporum}) properties (Bashyal et al., 2006; Suryanarayanan et al., 2009b), as well as growth promoting with phytohormone properties (Schulz et al., 2002). Thus, the fact that endophytes can produce secondary metabolites with biocidal properties make them attractive to the pharmaceutical industry (Schulz et al., 2002).
As with fungal endophytes, a number of low molecular weight compounds active at low concentrations against plant pathogenic fungi, animals and humans have been isolated from bacterial endophytes. For example, the peptides, Fengycin, Bacillomycin D, Zwittermicin A, Iturin A, Surfactin exhibit a strong antimicrobial activity produced by *Bacillus* species. An exhaustive list of antimicrobial compounds produced by different bacterial endophytes are described in Ryan et al. 2008.

2.6 Common techniques for observation, isolation and characterization of endophyte

In order to assess the viability of endophytes, some methods exist for detection, identification and isolation of endophytes from plant tissues.

2.6.1. Histological staining and tissue observation.

This technique involves observing the endophyte structures within the plant tissues using light microscopy. It is not the ideal method for the detection of bacterial endophytes. The recent application of green fluorescent protein (GFP) for tagging bacterial or fungal endophyte is a more precise way to visualize the presence of the endophyte *in situ*. With the help of specific vectors, it is possible to introduce the GFP gene into other organisms. This gene produces a protein when exposed to blue light produces a bright green fluorescent which can be visualized using fluorescence microscopy to detect the presence of endophyte inside plant tissue. Vectors such as pHRGFPT, pHRGFPGU have been used to
transform endophytic bacteria (Ramos et al., 2007; Rouws et al., 2010). Fungal endophytes can also be transformed using specific pPd-EGF vector (Mukherjee et al., 2010).

2.6.2 Surface sterilization

The host tissue is subjected to surface sterilization techniques followed by isolation of the endophytes on specific synthetic growth medium. The most common procedure employs a surfactant such as ethanol, followed by a sterilizing agent, such as sodium hypochlorite (Schulz and Boyle, 2005). The efficiency of sterilization is confirmed by the imprint technique. The technique consists of imprinting the plant tissue before and after sterilization on different growth media such as Malt Peptone Yeast Agar, Malt Extract Agar (MEA) (Arnold et al., 2000), Potato Dextrose Agar (PDA) (Latch and Christensen, 1985). All these media support the growth of fungal endophytes and are often amended with antibiotics in order to prevent bacterial endophytes growth (Schulz et al., 1999). For bacterial endophytes, a wide range of media are used for growth; Nutrient Agar (NA), Viande-Levure (Pavlo et al., 2011) or Rice extract Modified Rennie (RMR) are just some examples (Miyamoto et al., 2004). Once successfully isolated, endophytes are then sub-cultured, purified and maintained under controlled conditions for future applications.

2.6.3 Maintenance and culture of isolated endophytes
Fungal endophytes growing from the leaf section after sterilization are sub-cultured in order to obtain a pure culture, using the same media that they have been isolated on (MEA and PDA amended with antibiotics). The cultures are then incubated at 24°C. Once the fungus covers approximately ¾ of the plate, 5mm diameter punch holes are removed from the edge of the colony which represents the younger part of the fungal growth. Twenty plugs are place in a 2 ml sterile screw-cap tube, and covered with a sterile solution of Glycerol at a concentration of 25% (Kitamoto et al., 2002). The tubes are then frozen in liquid nitrogen and stored at -80°C.

Bacterial endophytes require to pass through a series of 4 single colony isolations to ensure their purity. Pure bacterial colonies are grown on LB (1.0% tryptone (Difco, Texas), 0.5% Yeast Extract (Difco, Texas), 1.0% and NaCl for 18 hours. An aliquot of 750 μl of bacterial solution are then pipetted into a 2 ml sterile screw-cap tube and mixed with sterile glycerol to obtain a 25% final glycerol solution. The tubes are then frozen in liquid nitrogen and stored at -80°C (Costa and Ferreira, 1991).

2.6.4 Detection

Fungal and bacterial endophytes in surface sterilized tissues can be detected by polymerase chain reaction methods (PCR) using the universal primers ITS 1 and ITS 4 encoding the ribosomal DNA space genes in fungi (Schulz and Boyle, 2005), or the 16sRNA
and ITS 16 (Miyamoto et al., 2004) encoding a spacer section in the ribosomal DNA of bacteria.

2.6.5 Identification using morphological parameters

In order to identify the endophytes, different techniques can be used. For fungi, condidiogenesis and spore morphology are both acceptable methods of classification (Arnold et al., 2000; Schulz and Boyle, 2005). For the non-sporulating fungi, the morpho-species method is used. This method is based on the observation of growth and morphological characteristic (Arnold et al., 2000). Identification of bacterial endophytes can be accomplished by morphological characteristics such as color, form, texture opacity as well as gram staining (Zinniel et al., 2002).

2.6.6 Identification using molecular markers

Molecular techniques such as the polymerase chain reaction (PCR) has proved powerful in detecting DNA of symbionts or endophytes that can not be cultured and separated from their co-symbionts (Haddad et al., 1995). Ribosomal DNA is now widely employed for estimating the phylogenies of various organisms followed by sequencing of the amplified product and homology comparison using Genbank or other database for identification (Arnold et al., 2000; Chiang et al., 2001).
The primer sequences of the internal transcribed spacer region of the nuclear ribosomal DNA (Sessitsch et al., 2004) is widely used for resolving phylogenetic relationships at the species or generic levels (White et al., 1990). Employing phylogenetic analysis is a common method for fungal and bacterial endophyte identification (Chiang et al., 2001; Larran et al., 2002; Mendes et al., 2007; Pavlo et al., 2011; Sun et al., 2008).
Chapter III Materials and Methods

3.1 Cultivars of Switchgrass

The cultivar, Cave-in-Rock originated from Southern Illinois and is the most recommended and widely used cultivar in North-Eastern America (Lemus et al., 2002). Cave-in-Rock evolved in a humid climate, for this reason it is well adapted to wet environment. Three other cultivars were also screened for the presence endophytes. Blue Jacket, an upland ecotype evolved from Sunburst and originated from South Nebraska (Boe and Ross, 1998; Samsons, 2011), Tecumseh, another upland ecotype that evolved from Summer and originated from South Dakota (Elbersen et al., 2001; Samsons, 2011), and Sand lover, an upland ecotype evolved cultivar NU 94-2 which originated from South Dakota (Samsons, 2011; Taliaferro, 2002). These three cultivars are well adapted to dry growth condition.

3.2 Field sites and sample selection

Two field sites in Valleyfield, Qc, Canada were selected for switchgrass (Panicum virgatum L.) collection. Field site 1 (45’ 16’ 29” N and 74° 4’2” W) was seeded in 1995 with cultivar Cave-in-Rock and received no fertilizer amendments until 2006 after which, only
50 kg N/ha was annually applied. Field site 2 (45°16′23″ N and 74°0′59″ W) was seeded in 2006 with cultivars Sand lover, Tecumseh and Blue Jacket and received no fertilizers in 2006 and 2007, but they received 50 kg /ha of N in 2008 and 2009. Both field sites were annually mowed in the fall and the material was baled in the spring.

Seeds from switchgrass plants showing the best agronomical traits from the 4 cultivars were collected on October 28, 2009 from both field sites, placed in envelopes and stored in the dark at room temperature. A portion of the seeds was reserved for DNA extraction, while the remaining seeds (912 seeds/cultivar) were planted on March 18th 2010 in 38-cells trays (Plant products Co. Ltd) containing 50 Pro-Mix HP /50 Pro-mix BX of potting mixture from of Agro mix® (Plant products Co. Ltd) , grown in a greenhouse at temperatures of 21°/19° day/night and under spring light conditions, and watered 3 times a week. Plant height was recorded after two months of growth. The tallest 20 plants of each cultivar were selected, transplanted into larger pots (10-cm diameter) containing the same substrate and after one month, they were transferred into field site 2 in Valleyfield on June 11th 2010. Plots were designed as a grid pattern (8.25 m²), in which there is 50 cm between plants on each axis

3.3 Sampling and plant sample processing

Plants were collected over two growing seasons in 2010 and 2011. At each sampling date (September 2010 and October 2010) four tillers per cultivar originated from seed
were grown and collected from different parts of the field and GPS coordinates were recorded and used for subsequent sampling. Additionally, tillers of 25 Cave-in-Rock switchgrass plants were collected in October 2011 from the oldest and established switchgrass field (Site 1). Leaves of eight (4 leaves per sampling date) asymptomatic plants (i.e., from seed grown switchgrass plants) of each cultivar were randomly sampled at two defined growth stages: late vegetative stage (i.e., the leaf at the upper node) and at full reproductive stage (i.e., the flag leaf) in the months of September and October 2010, respectively. A total of 25 flag leaves of Cave-in Rock from the established field were sampled. All samples were processed for bacterial and fungal endophytes. One leaf of each plant/cultivar/stage was sampled and immediately stored in individual Ziploc® bags, transported to the lab and processed within 48 hours.

Leaves were surface sterilized by step-wise washing in 99% ethanol for 1 min, rinsed in sterile water for 1 min, then immersed in a 5% solution of sodium hypochlorite for 5 min, followed by a rinse in sterilized water for 1 min then immersed in 99% ethanol for one min, and followed by three rinses (one min. each) in sterile distilled water (Schulz et al., 1993). The grass leaf was then separated into leaf sheath and leaf blade using a sterile blade, and each were cut into several 1-cm section pieces. Sections from each tissue were plated onto Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA) (Difco, Texas) at pH of 5.6 and 6.0 respectively amended with 60 mg l⁻¹ penicillin G + 80mg l⁻¹ streptomycin.
sulphate + 50 mg l⁻¹ chloromphenicol or onto Nutrient Agar (NA) (BBL, New-York), and incubated at 24°C in the dark for 4-6 weeks. The remaining leaf sections were transferred into small tubes, immersed in liquid nitrogen and kept in a -80°C freezer for genomic DNA extraction.

Seeds (1 gram) of the four cultivars were also subjected for surface sterilization method according Sauer and Burroughs (1986). Briefly, seeds were soaked in 5% solution of sodium hypochlorite for 20 min. with continuous stirring using an electromagnet. The seeds were subjected to three rinses of one min each in sterile distilled. Seeds were ground with liquid nitrogen and stored at -80°C for DNA extraction.

3.4 Efficacy of sterilization

The efficiency of surface sterilization procedure was ascertained following the imprint method of Schulz et al. (1993). In each Petri dish, 3 segments/tissue prior and after surface sterilization were imprinted for 5-10 seconds by carefully pressing the leaf sections onto antibiotic amended PDA, MEA and NA. The dishes were sealed with parafilm and incubated at 24°C ± 2 C for 4-6 weeks in dark. If there were microbes appearing on the imprinted culture plate after sterilization, the tissues were discarded.

3.5 Isolation and maintenance of endophytes
Cultures were examined regularly for emerging fungal and bacterial colonies. Emerging fungal colonies were passed through two rounds of sub-culturing on the same media in which they were isolated prior to long-term storage method according to Kitamoto et al. (2002). Briefly, 20 plugs (5 mm in diameter) were taken from 7 day-old fungal cultures, placed into screw-capped 2 ml tubes, covered with 25% solution of sterile glycerol, immersed immediately in liquid nitrogen and stored at -80°C. Emerging bacterial colonies were also passed through 4 rounds of single colony isolation by streaking them on NA culture medium to ensure purity of the organism prior to long term storage according to the method of Costa and Ferreira (1991). Briefly, single colonies were grown on LB (1.0% tryptone; 0.5% Yeast Extract, and 1.0% NaCl) for 18 hours. An aliquot of 750 μl of bacterial solution was placed in 2 ml sterile screw-caped tube and mixed with 25% glycerol solution. The tubes were flash frozen in liquid nitrogen then stored at -80°C

3.6 Identification of fungal and bacterial isolates

Sporulating fungi were identified based on colony morphology, conidiosphore and conidia morphology (Ellis, 1971). Isolated endophyte that failed to sporulate were broadly grouped by their macro-morphological characteristics. For bacterial endophytes, those were grouped on the basis of phenotypic characteristics, e.g., colony color and morphology, gram reaction staining (Steinbach and Shetty, 2001) and their antagonistic activity against selected fungi. All fungal groups were further refined and identified into taxa by DNA
cloning and sequencing of ITS regions. Bacterial strains that showed promising antagonistic activity against fungi were further grouped into their taxa based on ITS cloning and sequencing.

For further characterization of endophytes, 1x10⁹ of bacterial cells were harvested for genomic DNA extraction of test bacterial strains grown in LB broth for 18 hours with QIAGEN DNeasy® Blood & Tissues kit. Genomic DNA (100 mg) of test fungi grown on PDA covered with a cellophane membrane was extracted with QIAGEN DNeasy® Plant Mini kit and following the manufacturer’s recommendations. PCR amplification of the 16S rDNA gene for bacterial endophytes and of the ITS (ITS1, 5.8S, ITS2) coding sequence of fungal rDNA was performed with their respective universal primer set (Table 1), and was PCR amplified by using 20 ng genomic bacterial or fungal DNA. The PCR primers were used to sequence the purified PCR products. Briefly, 3 μl of the putative PCR products were cloned using the TOPO® TA-cloning Kit (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol. Plasmid DNA was purified using the PureLink™ Quick Plasmid Miniprep Kit (Invitrogen) and sent for sequencing at Genome Quebec (Montreal, QC). Gene sequences were manually inspected and edited into contigs using DNA sequence assembly using CAP3 Sequence Assembly Program. Sequences were then subjected to Blastn searches against NCBI database. The top 5 hits, with the lowest e-value, were used to assign identity. The nucleotide sequences were deposited to GenBank public database (Table 1)
3.7 Species-specific primer design and phylogenetic analysis

The most similar sequences of endophytic fungi and bacteria were further aligned using ClustalW software in SDSC Biology Workbench (Subramaniam, 1998) and the non-conserved regions were used to design specific primers for each endophytic fungus and bacterium. Specific primers were synthesized by Integrated DNA Technologies Inc (Coralville, Iowa USA), and were tested against all fungi and bacteria to insure specificity.

To construct phylogenetic trees for bacterial endophytes, the nucleotide sequence of each bacterial endophyte was aligned with sequences of selected known strains of bacteria using ClustalW software, and the trees for bacterial endophytes were built using MEGA 5.05 software (Tamura et al., 2011). Maximum likelihood method was employed to infer the tree topology. The reliability of the trees was tested by bootstrapping 1,000 replicates generated with a random seed.

3.8 Detection of fungal and bacterial endophytes using PCR methods- plants and seeds

Sterilized switchgrass plant tissues (i.e., sheaths and blades of leaf stages and seeds) were reduced to powder under liquid nitrogen using a mortar and pestle and subjected to DNA extraction extracted using the Dneasy Plant Mini kit 50 (GIAGEN®, Ontario). The
presence of endophytes within switchgrass tissues was confirmed by PCR using the GeneAmp® PCR System 9700 (Applied Biosystem, California). Each amplification mixture contained 2.5 μl of 10x PCR buffer (Fermantas®, Ontario, Ontario), 2.5 μl of dNTP (2mM), 2 μl of MgCl₂ (15mM), 1.5 μl of each primer (2mM), 0.5 U of Taq polymerase (Fermantas®, Ontario), and 8μl of template DNA (5ng/μl) in a total volume of 25μl. All PCR reactions were run under the following conditions: one cycle of initial denaturation at 94°C for 10 min., followed by 35 cycles of denaturation at 95°C for 45 s, annealing for 30 s at specific temperatures (Table 1), and extension at 72°C for 45 s. The program ended with an additional 7 min and additional extension at 72°C followed by a cool down to 4°C. All primer sets were run with a positive control and a negative control containing no template DNA. All samples were run on a 1% agarose (Applied Biological Materials, Inc., B.C., Cat # GO60-2) gel electrophoresis and visualized using Gel Logic 200 Imaging system® from Mendel under U.V. light.

3.9 Antagonistic study by dual culture method

Twenty-one bacterial endophyte isolates were screened for antifungal activity by dual culture method against plant pathogenic fungi and biological control agents that were obtained from established fungal data banks and from collaborators (Table 3). Bacterial endophytes and test fungi were grown on PDA culture plates and incubated at 24°C. A 5mm diameter mycelial plug taken from the edge of actively-growing test fungus was placed in
the middle of the culture plate containing 15 ml of PDA. A 10μl aliquot of LB broth containing bacterial of suspension with a concentration of 10^5 CFU ml^-1 was aseptically deposited on both sides at 2.5 cm from test fungus. Simultaneously, culture plates inoculated with test fungi and the endophytes served as control. All plates were allowed to grow at 24°C in the dark for 5 days. Three replicates were used for each test fungus. The inhibitory effect on fungal growth was evaluated by development of an inhibition zone on either side of the test fungus and compared with control (fungus alone).

3.10 Inoculation of switchgrass seedling with Bacterial endophyte isolate and confirmation of the re-isolated bacteria.

To test whether bacterial endophytes were able to colonize switchgrass, bacterial isolates were introduced into seedling grown aseptically under controlled conditions. Magenta® GA-7 Plant Culture Boxes 3 x 3 x 4" (Magenta, Chicago III) containing 100 g of sand and vermiculite (50/50% v/v) were autoclaved for 1 h every 24 h for a period of 72 h. Twenty surface sterilized switchgrass seeds were seeded in each Magenta box and grown in a growth chamber at 22°C under a 12-h/12-h of light/dark cycle. Each box received 5 ml of sterile-distilled water at seeding. Bacterial endophytes were grown in LB broth for 24 hours to the mid-log phase, pelleted by centrifugation, washed and suspended in sterile distilled water. After two weeks, plants were thinned down to 15 seedlings, which received
5 ml of water containing $10^5$ CFU/ml of bacteria. Switchgrass seedlings receiving autoclaved distilled water served as control. Putative endophytes *Bacillus subtilis* and *Microbacterium testacum* were tested alone and in combination. Plants were incubated further for another 2 weeks. Four-week-old seedlings were dipped in a solution of 70% ethanol, rinsed in autoclaved-distilled water, separated into roots and shoots, and subjected to DNA extraction using QIAGEN DNeasy® Plant Mini kit. Using specific primers designed for each endophyte, the presence of endophytes was assessed in roots and shoots of inoculated and non-inoculated switchgrass. The experiment was replicated 8 times and each replicate contained 15 plants.
Chapter IV Results

4.1 Efficacy of sterilization

The surface sterilization protocol was a critical prerequisite for isolating plant endophytic bacteria and fungi. This study proved that the surface sterilization protocol combined with the imprint technique was effective in removing epiphytic organisms and that the bacterial and fungal isolated strains can be considered to be true endophytic organisms.

4.2 Survey of Switchgrass endophytes and molecular identification

Over the course of this study (2010 and 2011), 594 switchgrass leaf segments were incubated and the total number of culturable endophytes was 145 among which 93 strains were fungi and 52 strains were bacteria (Figures 1A-B). The majority of the fungal (91%) and bacterial (73%) endophytes were recovered from the switchgrass cultivar Cave-in-Rock. Of those, 80 fungal and 26 bacterial isolates originated from old stands of Cave in
Rock, followed by those recovered from Blue Jacket, while the remaining endophytes were distributed equally between Tecumseh and Sand Lover (Figures 4.1A-4.1B). Interestingly, irrespective of the cultivar or leaf type (i.e., vegetative or reproductive), 75 out of 93 fungal endophytes originated from switchgrass blades, while bacterial endophytes were almost equally distributed between the sheaths and the blades (Figures 4.1C-1D).

The fungal isolates were grouped into 9 morphogenic groups and were identified into different taxa based on cloning and sequencing of amplified fragments (300-700 bp) of the ITS region (Table 4.1). All isolates showed a good homology ranging from 93 to 100% with other known sequences (Table 4.2) and partial sequence data for the 18s rDNA have been deposited in the GenBank (NCBI) nucleotide sequences data base library. Data for endophytic strains have been deposited under the following accession numbers (JN689341-JN689349). The fungi (Table 4.2) were identified as Chaetomium globosum (Sordiales) Epicoccum nigrum (Dothideales), Emericella spp. (Eurotiales), Ascochyta sp. (Sphaeropsidales), Penicillium resedanum (Eurotiales), Alternaria alternata (Pleosporales), Aspergillus versicolor (Eurotiales), Cladosporium tassiana (Mucorales), and Syncephalastrum racemosum (Mucorales). All of which are known as common fungal endophytes. C. globosum, E. nigrum and Ascochyta sp. were the most widely isolated strains (data not shown).
Twenty-two of the bacterial isolates tested gram-positive and 30 were gram-negative. A total of twenty-one strains of bacterial endophytes were randomly selected out of 52 isolated strains and tested for their antagonistic activities against selected pathogenic fungi in dual culture assays (Figure 4.2). Strains showing antagonistic activity were subsequently identified by cloning and sequencing the amplified fragment (1505 bp) using the ITS sequence data. Also, two additional strains that did not exhibit any antagonistic activity were also sequenced. Partial sequence data for the 16s rDNA gene have been deposited in the GenBank (NCBI) nucleotide sequences data base library. Data for endophytic strains have been deposited under the following accession numbers (JN689336-JN689340). All were grouped into 5 different taxa that shared high homology of 98-99% with other known sequences (Table 4.2). Bacterial endophytes were identified as *Microbacterium testaceum* (Gram-positive; Actinomycetales), *Curtobacterium flaccumfaciens* (Gram-positive; Actinomycetales), *Pseudomonas fluorescens* (Gram-negative; Pseudomonadales), *Bacillus subtilis* (Gram-positive; Bacillales) and *Panteoa ananatis* (Gram-negative; Enterobacterales), with the latter as the most frequently isolated endophyte (data not shown).

Phylogeny analysis based on a maximum likelihood with a bootstrap analysis repeated 1000 times was performed on the identified using the bacterial 16S rRNA gene sequences. The analysis revealed a good homology. For example, the homology value
between the isolate *Panteoa ananatis* JN689340 and *Pantoea ananatis* GQ383910 from Genbank was 99%. The rooted maximum likelihood tree showed good bootstrap values at the nodes many of them above 70%, which demonstrate the most probable branching of the tree. Each of the isolated endophyte clustered with a single group of species, which indicate the good identification of the organism.

4.3 Detection of fungal and bacterial endophytes using PCR methods- plants and seeds

The presence of the identified endophytes in various tissues (leaves and seeds) of field-grown switchgrass cultivars was confirmed by PCR assays using species-specific designed primer for each one of the identified endophytes. The presence of endophytes varied with cultivars and tissue types. The bacterial endophytes, *B. subtilis* and *C. flaccumfactiens*, and the fungal endophytes, *E. nigrum*, *Ascochyta* sp., *S. racemosum* and *P. resedanum* were detected in tissues of switchgrass grown from seeds collected in 2009 (Figure 4.4). Others were detected in one switchgrass cultivar only such as, the bacterial endophytes *M. testacum* and the fungal endophytes *C. globosum*, *A. versicolor* were found in Cave-in Rock cultivar. The fungal endophyte *Emericella* sp. was found in Tecumseh and Blue Jacket. The species-specific primer sets failed to detect the bacterial endophytes: *Pseudomonas fluorescens*, *Panteoa ananatis* and fungal endophytes *A. alternata* and *Cladosporium tassiana* in field switchgrass although they successfully amplified the endophyets when grown in pure culture (Data not shown). Interestingly, vertical
transmission of the following endophytes via seeds; *B. subtilis*, *E. nigrum*, *Ascochyta* sp., *P. resedanum*, and *S. racemosum* was confirmed in switchgrass grown in 2010 and originated from seeds collected in 2009 (Figure 4.5D).

4.4 Antagonistic property of endophytes and re-inoculation of switchgrass with endophytes

Six out of 21 bacterial strains were antagonistic against all different test fungi. Antagonism towards the test fungi was recorded as inhibition zone developing during dual culture and ranging from 1 mm to greater than 3 mm (Figures 4.2A and B; Table 4.3). *Bacillus subtilis* (strains B26 and B32) and *P. fluorescens* (strain B25) were effective against all test fungi, followed by *P. ananatis* (strains B45, B46 and B47) that was antagonistic against selected test fungi.

The presence of endophytes was successfully detected in roots and shoots of four-week-old switchgrass inoculated singly (Figures 4.5A and 4.5B) or in combination with *Bacillus subtilis* and *Microbacterium testacum* (Figures 4.5C). Absence of endophytes was confirmed in non-inoculated switchgrass seedlings (Figures 4.5A and 4.5B).
Table 4.1. List of isolated endophytes, specific and universal primers designed and used in polymerase chain reaction (PCR) assays

<table>
<thead>
<tr>
<th>Targeted organism</th>
<th>Forward and reverse primer sequences (5' to 3')</th>
<th>Primer Tm(°C)</th>
<th>PCR product size</th>
<th>Genebank accession number for targeted gene</th>
<th>Reference</th>
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<td><em>Microbacterium testaceum</em></td>
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<td>850</td>
<td>JN689338</td>
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<tr>
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<td>Accession Number 1</td>
<td>Organism</td>
<td>Sequence 2</td>
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<tr>
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<td><em>Ascochyta</em> sp.</td>
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<td></td>
<td>JN689346</td>
<td><em>Aspergillus versicolor</em></td>
<td>CTAACACTGTGGCTTCGGCGGG</td>
</tr>
<tr>
<td></td>
<td>CTCTGAACCCTGTTCTGAAGTAAG</td>
<td></td>
<td></td>
<td></td>
<td>ATACGCTCGAAGGCCGACAC</td>
</tr>
<tr>
<td><em>Syncephalastrum racemosum</em></td>
<td>GAAGACACICTTAGCGCACGCA</td>
<td>64</td>
<td>JN689349</td>
<td><strong>Universal primer</strong></td>
<td><strong>Bacterial universal primers</strong></td>
</tr>
<tr>
<td></td>
<td>CAGCGCAGGGGCAATCATA</td>
<td></td>
<td></td>
<td></td>
<td>(1492f/27r)</td>
</tr>
<tr>
<td></td>
<td>AGAGTTTGATCMTGGCTCAG</td>
<td>58</td>
<td>1465</td>
<td></td>
<td>GGTTACCTTGTTACGACT</td>
</tr>
<tr>
<td></td>
<td>TCCGTAGGTGAACCTTGCGG</td>
<td></td>
<td></td>
<td></td>
<td>TCCTCCGCTTATGTATGC</td>
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</table>
Plant universal primer  
CATTACAATGCAGATGCCTCT 
TCTACCGATTTCCGATATC 

55 300-700 11 

Table 4.2. Molecular identification of bacterial and fungal endophytes from *Panicum virgatum* L. based on blastN queries in NCBI

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Switchgrass Cultivar</th>
<th>Genbanck accession No.</th>
<th>Closest blast match (Genbanck accession No.)</th>
<th>Query/reference ITS length (Similarity %)</th>
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</thead>
<tbody>
<tr>
<td><strong>BACTERIA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Curtobacterium flaccumfaciens</td>
<td>Cave-in-Rock</td>
<td>JN689336</td>
<td><em>Curtobacterium flaccumfaciens</em> (AM410688)</td>
<td>1502/1504 (99)</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>Cave-in-Rock</td>
<td>JN689337</td>
<td><em>Pseudomonas fluorescens</em> (DQ439976)</td>
<td>1476/1500 (98)</td>
</tr>
<tr>
<td>Microbacterium testacum</td>
<td>Cave-in-Rock</td>
<td>JN689338</td>
<td><em>Microbacterium testacum</em> (EU714365)</td>
<td>1455/1466 (99)</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>Cave-in-Rock</td>
<td>JN689339</td>
<td><em>Bacillus subtilis</em> (HQ727971)</td>
<td>1502/1503 (99)</td>
</tr>
<tr>
<td>Pantoea ananatis</td>
<td>Cave-in-Rock</td>
<td>JN689340</td>
<td><em>Pantoea ananatis</em> (GQ383910)</td>
<td>1483/1490 (99)</td>
</tr>
<tr>
<td><strong>FUNGI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chaetomium globosum</td>
<td>Cave-in-Rock</td>
<td>JN689341</td>
<td><em>Chaetomium globosum</em> (HQ529775)</td>
<td>575/576 (99)</td>
</tr>
<tr>
<td>Epicoccum nigrum</td>
<td>Cave-in-Rock and Tecumseh</td>
<td>JN689342</td>
<td><em>Epicoccum nigrum</em> (FN868456)</td>
<td>576/576 (100)</td>
</tr>
<tr>
<td>Emericella sp.</td>
<td>Cave-in-Rock</td>
<td>JN689343</td>
<td><em>Emericella sp.</em> (AB249015)</td>
<td>581/581 (100)</td>
</tr>
<tr>
<td>Ascochyta sp.</td>
<td>Cave-in-Rock</td>
<td>JN689344</td>
<td><em>Ascochyta hordei</em> (HQ882800)</td>
<td>479/490 (98)</td>
</tr>
<tr>
<td>Penicillium resedanum</td>
<td>Cave-in-Rock</td>
<td>JN689445</td>
<td><em>Penicillium resedanum</em> (AF033398)</td>
<td>576/579 (99)</td>
</tr>
<tr>
<td>Alternaria alternata</td>
<td>Cave-in-Rock</td>
<td>JN689446</td>
<td><em>Alternaria alternata</em> (JN618076)</td>
<td>423/423 (100)</td>
</tr>
<tr>
<td>Species</td>
<td>Location</td>
<td>Accession Number</td>
<td>Description</td>
<td>Identity Percentage</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>--------------</td>
<td>------------------</td>
<td>----------------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td><em>Aspergillus versicolor</em></td>
<td>Cave-in-Rock</td>
<td>JN689447</td>
<td><em>Aspergillus versicolor</em> (AY373880)</td>
<td>568/569 (99)</td>
</tr>
<tr>
<td><em>Cladosporium tassiana</em></td>
<td>Tecumseh</td>
<td>JN689348</td>
<td><em>Cladosporium cladosporioides</em> (HQ380766)</td>
<td>548/548 (99)</td>
</tr>
<tr>
<td><em>Syncephalastrum racemosum</em></td>
<td>Cave-in-Rock</td>
<td>JN689349</td>
<td><em>Syncephalastrum racemosum</em> (HQ2855713)</td>
<td>303/326 (93)</td>
</tr>
</tbody>
</table>
Table 4.3. Dual culture antagonistic experiment using different fungi

<table>
<thead>
<tr>
<th>Test fungus</th>
<th>B4</th>
<th>B8</th>
<th>B25</th>
<th>B26</th>
<th>B32</th>
<th>B45</th>
<th>B46</th>
<th>B47S</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fusarium solani</em>(^1)</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Binucleate rhizoctonia</em> 1707(^2)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Rhizoctonia solani</em>(^3)</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><em>Trichoderma virens</em>(^4)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Stachybotrytis elegens</em>(^4)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><em>Phytophthora infestans</em>(^1)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Verticillium albo atrum</em>(^1)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

No inhibition; +, zone of inhibition 1 to 3 mm; ++, zone of inhibition of more than 3 mm $ numbers in bracket represent the strain designation

1. G. Lazarovits, Agriculture, Agri-Food Canada, London, Ontario, Canada; 2. S. Neate, DPI, Queensland, Australia; 3. M.A. Cubeta, CIFR, North Carolina State University, NC, USA; 4. American Type Culture Collection (ATTC), Manassas, VA20108, USA.
Fig. 4.1. Distribution of fungal and bacterial endophytes across the different cultivars and leaf tissues. (A) Fungal isolates across the different cultivars (B) Bacterial isolates across the different cultivar (C) Fungal endophytes in different leaf tissues (D) Bacterial endophytes in the different leaf tissues.
Fig.4.2. Antagonistic test of (A) *Pantoea ananatis* and (B) *Bacillus subtilis* against *Rhizoctonia solani*.  
- **(A-1)** R. solani;  
- **(A-2 and A-3)** R. solani and *P. ananatis*;  
- **(B-1)** Rhizoctonia solani alone;  
- **(B-2 and B-3)** Rhizoctonia solani and *Bacillus subtilis*. 


**Fig.4.3.** Maximum likelihood phylogenic tree of all putative endophytic bacteria. The tree based analysis of partial 16S rDNA sequences of bacterial isolates from switchgrass. Numbers above each node indicate percentage of confidence levels generated from 1000 bootstrap trees and the GeneBank Accession numbers precede the species names.
Fig. 4. Example of PCR detection of fungal (A) and bacterial (B) endophytes detection in different switchgrass cultivars using species-specific primers. (A) *Ascochyta* sp. and (B) *Bacillus subtilis*. 100 bp DNA ladder lanes 1, lanes 2 Pure fungal genomic DNA of (A) *Ascochyta* or (B) *Bacillus subtilis* lanes 2, No template lanes 3, PCR on template isolated from leaves of Tecumseh lanes 4-7, Cave-in-Rock lanes 8-11, Blue Jacket lanes 12-15, Sand Lover lanes 16-19, PCR on template isolated from seeds of Tecumseh, Cave-in-Rock and Blue Jacket, respectively lanes 20-21.
Fig. 5. PCR detection of *Bacillus subtilis* (A), *Microbacterium testaceum* (B), the combination of both (C) recovered from colonized switchgrass seedlings. (D) example of vertical transmission of the fungal endophyte *Epicoccum nigrum*. (A) 100 bp DNA ladder lane 1, Pure DNA of *B. subtilis* lane 2, No template lane 3, DNA of non-inoculated switchgrass seedlings lanes 4-6, DNA of re-inoculated switchgrass seedling with *B. subtilis* lane 7, DNA from roots of colonized switchgrass lane 8, DNA from leaves and stems of colonized switchgrass lane 9; (B) 100 bp DNA ladder lane 1, DNA of non-inoculated switchgrass seedlings lane 2-5, DNA from roots of colonized *M. testaceum* switchgrass seedling lanes 6-7, DNA from stems and leaves of colonized *M. testaceum* switchgrass lanes 8-9, Pure genomic DNA of *M. testaceum* lane 10, No template lane 11; (C) 100 bp DNA ladder lane 1, Pure genomic DNA of *B. subtilis* lane 2, No template lane 3, DNA from roots of colonized switchgrass with *B. subtilis* and *M. testaceum* and tested with *B. subtilis* primers lane 4, DNA from shoots of colonized switchgrass with *B. subtilis* and *M. testaceum* switchgrass and tested with *B. subtilis* primers lane 5, pure genomic DNA of.
B. subtilis lane 6, No template lane 7, DNA from roots of colonized switchgrass with B. subtilis and M. testaceum switchgrass and tested with M. testaceum primers lane 8, DNA from shoots of colonized switchgrass with B. subtilis and M. testaceum and tested with M. testaceum primers lane 9; (D) 100 bp DNA ladder lane 1, Pure genomic DNA of E. nigrum lane 2, No template lane 3, DNA of flag leaf collection from cultivar Cave-in-Rock line 4, collected seeds from cultivar Cave-in-Rock line 5, flag leaf from Cave-in-Rock grown from seed lane 6.
Chapter V Discussion

Endophytic fungi and bacteria are ubiquitous in nature, infecting virtually all plants in both natural and in agronomic ecosystems (Hyde and Soytong, 2008; Ryan et al., 2008; Schulz and Boyle, 2005). Some of these likely have no beneficial effects on host fitness and may be either latent pathogens or saprophytes that remain inactive until environmental cues trigger a developmental shift for the fungus or bacteria to continue its lifecycle. Others have been repeatedly isolated and described as endophyte and showed to be beneficial in some way to the plant. The endophytes isolated over the course of this work were obtained using a traditional method, in which only endophytes that can grow on microbiological media were accounted for which may create a negative bias towards endophytes that are slow growing or that can’t grow on biological media. In order to assess the full range of endophyte future work should incorporate techniques like DGGE or T-RLFP (Bridge and Newsham, 2009; Duong et al., 2006). As the over arching aim of this study is to identify endophyte species that could be introduced into elite switchgrass cultivars for maximizing their utility as a bionergy crop, we focused on these endophytes that could be grown on pure culture and by using different media with different sources of carbon and pH, we believe to have countered as many as possible.
Our research goals were to survey switchgrass grown in Quebec for the presence of endophytic bacteria and fungi, and to determine their taxonomic position. Strategically, in order to cover a maximum genetic diversity, we sampled leaves from old and well-established stand as well as from recently established fields of four switchgrass cultivars having different genetic background. In this study, we demonstrated that a wide range of fungal and bacterial species from 11 different taxa orders exist as endophytes in switchgrass cultivars. Similarly, other researchers have reported on the isolation of indigenous bacterial endophytes from leaf tissue (Zinniel et al., 2002) and fungal endophytes from leaf and root tissues (Ghimire et al., 2011) of switchgrass that naturally inhabits prairies. To the best of our knowledge, our study is the first to describe both indigenous fungal and bacterial endophytes from elite switchgrass cultivars produced through breeding. There appears to be significant variation in the types of indigenous fungi and bacteria from naturally occurring and breeding lines of switchgrass. The only common fungal endophyte recovered from prairie switchgrass (Ghimire et al., 2011) and from the switchgrass breeding lines (our study) was *A. alternata* and *Ascochyta* sp. (anamorph *Didymella*). In the case of bacterial endophytes, *Curtobacterium* and *Microbacterium* species were common as they were recovered endophytes from prairie switchgrass (Zinniel et al., 2002) and from the cultivars used in this study. Several factors may explain these differences, including cultivar type, plant age and tissue type and seasonality (Clay, 1984; Ghimire et al., 2011)
Some of the fungal genera to which our switchgrass isolates have been assigned are known to display a variety of interactions with plants. For instance, *Alternaria alternata* has been described a frequently recovered endophyte (Guo et al., 2004) as well as a plant pathogen causing post-harvest diseases of fruits and vegetables (Tian et al., 2011). Another fungal genus, *Cladosporium* exhibits interactions with plants ranging from facultative biotrophs to endophytes and epiphytes (Wirsel et al., 2001) and has been reported along with *Aspergillus versicolor* to display anti-micorbial activity against several human pathogens (Sette et al., 2006). We are currently investigating these isolates in details to establish their physiological capacities.

Over the course of this study, endophytes were isolated from 594 switchgrass leaf segments with the majority (85%) of bacterial and fungal endophytes originating from an old stand of Cave-in-Rock cultivar that was established sixteen years ago. This is not surprising as the disparity in the number of isolates per cultivar may be the result of plant age. As time of exposure to endophyte inoculum increases, plants seem to accumulate an increasing number of endophytes in their tissues. Because of this, older plant parts may harbor more and diverse populations of fungal and bacterial endophytes than younger ones (Arnold and Herre, 2003; Clay, 1984). Another plausible explanation for increased incidence of recovered endophytes from Cave-in-Rock may be related to the environmental
conditions favoring the incidence of endophytes. It has been shown that humid and hot conditions are likely more conducive for increased incidence of endophytes than arid and cold conditions (Arnold and Lutzoni, 2007; Sánchez Márquez et al., 2010). Among the switchgrass cultivars used in this study, cultivar Cave-in-Rock was developed in humid areas of southern Illinois, USA (Elbersen et al., 2001), and probably evolved to promote higher endophyte incidence as the plant under such conditions have boundless access with fungal endophytes and pathogens.

The findings of our study show that the distribution of fungal, but not bacterial endophytes, in different leaf parts was substantially unequal. We were able to isolate significantly more fungal strains from the leaf blade compared to the leaf sheath. We are not able to explain this observation, however recent studies have shown that the extent of leaf colonization by fungal endophytes including that of leaf blades varies among host grasses and probably reflects the stage of development of the primordial leaf when the hyphae become established (Christensen and Voise, 2009). Also, leaf colonization by fungal endohoytes is related to the timing of entry of the hyphae into developing leaves (Christensen et al., 2000). The ligular zone found just below the leaf blade lacks continuous intercellular spaces and acts as a physical barrier preventing the entry of hyphae into leaf blades (Hinton and Bacon, 1985). Although in our study, changes in endophyte distribution over time was not addressed, it is likely that leaf blade colonization of
switchgrass by fungal endophytes occurred before a certain stage in the
development of the ligular zone. Another plausible explanation is the
difference in the nature of the tissues composing the leaf sheath and the leaf
blade might explain the difference in the number of isolate. The leaf sheath
has a much greater content of lignified tissues than the leaf blade
(Rangasamy et al., 2009). Lignified tissues are harder to penetrate for fungi
and provide a natural plant protection, thus making it less hospitable for the
endophytes (Raven et al., 2005).

The dominant taxa recovered from switchgrass reflect an unequal
distribution of isolate richness among species. This kind of abundance has
also been observed in other grasses (Sánchez Márquez et al., 2010; Wirsel et
al., 2001). It is not yet clear whether this abundance inequality is method
related, that is selection of certain taxa by the cultivation method. Several
dominant taxa of Switchgrass endophytes also have been identified in other
grasses and plants of other families implying that these species are host‐
generalists. Examples of these taxa are Epicoccum, Chaetomium, Ascochyta
and Panteoa (Hashizume et al., 2010; Mano et al., 2007; Neubert et al., 2006;
Sánchez Márquez et al., 2010).

Our results demonstrated that the five different recovered taxa of
bacteria from switchgrass are also associated with grasses and plants as
endophytes (Lodewyckx et al., 2002; Zinniel et al., 2002). The beneficial
effects of bacterial endophytes on their host plants appear to occur through similar mechanisms described for plant-growth promoting rhizobacteria (Lodewyckx et al., 2002). Plant growth-promoting bacteria can affect plant growth directly or indirectly. The direct promotion of plant growth by PGPR or endophytes such as *Microbacterium testaceum* and *Panteoa ananatis* (Thomas et al., 2007; Zinniel et al., 2002) for the most part, entails providing the plant with a compound that is synthesized by the bacterium and the uptake of certain nutrients from the environment. The indirect promotion of plant growth occurs when PGPR or endophytes (e.g., *Pseudomonas fluorescens*, *Bacillus subtilis*, *Curtobacterium flaccumfaciens*, and *Panteoa species*) decrease or prevent the deleterious effects of one or more phytopathogenic organisms via release of antimicrobial metabolites (Bacon et al., 2001; Landa et al., 2004; Mano et al., 2007; Thomas et al., 2007).

Our ITS-sequence database for switchgrass-associated fungi was not only the basis for molecular taxonomy but also served as a source for designing species-specific primers for monitoring individual fungal species within DNA isolated from leaves and seeds of switchgrass cultivars. We have applied this approach for tracking individual endophytes by PCR analysis in seeds and of mixed endophytes reintroduced into switchgrass seedlings. Seed-borne endophytes can provide improved vigor to switchgrass, which could be most important when establishing new switchgrass stands. In our study, the endophytes *B. subtilis*, *E. nigrum*, *P. resedanum*, *S. racemosum* were
frequently detected in switchgrass seeds as well as plants grown in 2010 that originated from seeds collected in 2009. These results demonstrate that the endophytes are carried by seeds as a viable propagule and can be vertically transmitted to the next generation of the host. The same type of transmission is found in common reed, corn, coffee and some native grasses (Bacon et al., 2001; Ernst et al., 2003; Vega et al., 2006)

Endophytic bacteria inside a plant might either become localized at the point of entry or spread throughout the plant (Hallmann et al., 1997). These microorganisms reside within cells, in the intercellular space or in the vascular system (Hallmann et al., 1997). In our colonization experiments, B. subtilis and M. testaceum were recovered from sterilized roots, and leaves two weeks after inoculation of switchgrass seedlings, indicating successful internal colonization within the roots and the leaves. These results indicate that the endophytes in switchgrass seedlings moved from the roots and travelled upward to the stem and leaves. Our results concur with previous findings, which also demonstrate the ability of B. subtilis endophytic strain to enter the roots and migrate in the upper stems and leaves of eggplants (Lin et al., 2009).
Chapter VI Conclusion

In summary, we recovered 9 different endophytic fungi and 5 different endophytic bacteria from four switchgrass cultivars. These endophytes were identified using molecular techniques. Our findings are in agreement with other studies that the recovered identified endophyte are known endophytes in other plants, including perennial grasses.

The in-vitro dual-culture method using endophytic bacteria, demonstrated their bioactivity potential against plant pathogenic fungi. Our results corroborate the finding of several studies which show the antagonistic activity of bacterial endophytes. Thus, switchgrass endophytes represent a potential source of symbionts that can play an important role in switchgrass production.

The species-specific primers that we have developed can now be applied to systematically screen samples from various natural habitats, locations, and seasons in order to reveal putative correlations between switchgrass health and the switchgrass-associated endophytes discovered in this study.

The successful colonization of switchgrass with selected endophytes, either applied singly or in combination suggests that they can be utilized in
future applications such as delivery of degradative enzymes or antimicrobial metabolites for controlling diseases.
Chapter VII References


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