Factors influencing the incidence of Pythium stunt caused by *Pythium tracheiphilum* in head lettuce crops.

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ABSTRACT

In this study, the author intended to determine the factors, biotic and abiotic, influencing Pythium stunt (*Pythium tracheiphilum* Matta) incidence in head lettuce production. The first part of the study was two-fold. First, controlled environment trials were carried out to determine a threshold level of soil inoculum density for lettuce grown under fresh and moist conditions and in a soil that was previously heated to reduce its biological activity. Results obtain from this first part suggest that a concentration of 97 (cv. Estival) or 46 (cv. Prestige) spores per gram of dry soil was needed to reduce the lettuce growth by half. Second, a real time PCR assay was used to quantify the soil inoculum density in different lettuce fields of the Napierville county in order to determine the relationship between the inoculum density and the disease incidence under natural conditions. Air temperature, relative humidity and rainfall were also monitored and correlated with the disease. A binary recursive partitioning (BRP) analysis was carried out in order to set threshold values relative to each parameter. Results from this study suggest that, 79% of the time, a temperature below 18°C during the first two weeks after lettuce planting, a rain accumulation above 64 mm for the first three weeks as well as a soil inoculum density above 132 spores per gram of dry soil were accurate to predict a disease incidence of more than 5%.

In the second part of the study, metagenomic analysis was used to characterize the bacterial, eukaryotic and fungal composition of rhizosphere soil collected from healthy and diseased lettuce, and of lettuces grown in either a suppressive or a conducive soil to *P. tracheiphilum*. The microbial composition was first analysed using a principal coordinate analysis (PCoA) and the diversity was estimated using the Chao1 index and the Shannon

index. Taxonomic identity was also attributed to each OTUs amplified, at different taxonomic level and percentage of relative abundance for each sample were calculated. Results obtained from this second part of the study suggest that the microbial composition was similar when healthy and diseased lettuces were compared but was different between suppressive and conducive soils. Suppressive soil to *P. tracheiphilum*, in this study, was characterized by the presence of more Basidiomycota as well as more species known for either their plant growth promotion or their pathogen-antagonistic activities. Therefore, results suggest that the pathogenicity of *P. tracheiphilum* is modulated by biological factors.

RÉSUMÉ

Ce projet consiste en l'étude des facteurs, biotiques et abiotiques, pouvant influencer l'incidence de l'affaissement pythien (*Pythium tracheiphilum* Matta) dans la culture de laitues pommées. La première partie du projet avait deux objectifs principaux. Dans un premier temps, des expériences sous conditions contrôlées furent effectuées afin de déterminer une concentration d'inoculum seuil pour des laitues croissant dans des conditions fraîches et humides et dans un sol ayant préalablement été chauffé afin d'en réduire l'activité biologique. Les résultats obtenus dans cette première partie suggèrent qu'une concentration de 97 (cultivar Estival) ou 46 (cultivar Prestige) spores par gramme de sol sec est la quantité minimale requise afin de réduire de moitié la croissance des plants de laitues. Dans un second temps, des techniques de PCR en temps réel furent utilisées afin de quantifier l'inoculum de sol de différents champs de laitues commerciaux de la région des Jardins de Napierville, afin d'évaluer la relation entre l'inoculum du sol et l'incidence de la maladie sous conditions naturelles. Des données concernant la température de l'air, l'humidité relative ainsi que la pluviométrie furent également collectées et corrélées avec l'incidence de la maladie. Une analyse de partition binaire récursive (PBR) fut effectuée afin de déterminer des valeurs seuil relatives à chaque paramètre étudié. Les résultats de cette seconde partie suggèrent qu'une température inférieure à 18°C durant les deux premières semaines suivant la transplantation au champ, une accumulation de pluie de plus de 64 mm pour les trois semaines suivant la transplantation ainsi qu'un inoculum de sol de plus de 132 spores par gramme de sol sec peuvent être utilisé pour prédire correctement une incidence de la maladie de plus de 5%, et ce dans 79% des cas.

Dans la seconde partie du projet, des analyses métagénomique furent utilisées afin de caractériser la composition bactérienne, eucaryotique et fongique d'échantillons de sol provenant de la rhizosphère de laitue saines ou affectées par l'affaissement pythien, et de laitues ayant poussées dans un sol soit suppressif ou propice à *P. tracheiphilum*. La composition microbienne fut d'abord analysée à l'aide d'une analyse en coordonnées principales (PCoA) et la diversité microbienne fût estimée à l'aide des index de Chao1 et de Shannon. L'identification taxonomique relative à chaque OTUs amplifiés fut déterminé pour différent niveaux taxonomiques et des pourcentages d'abondance relative furent également calculés. Les résultats obtenus dans cette partie suggèrent premièrement que la composition microbienne entre les plants affectés ou sains est la même, mais qu'elle diffère lorsque les sols suppressifs et propices sont comparés. Les sols suppressifs dans cette étude furent caractérisés par une plus grande abondance des Basidiomycètes ainsi que par une plus grande présence d'espèces connus pour leurs propriétés bénéfiques pour la croissance des plantes ou pour leur activité d'antagoniste de pathogène. Ainsi, les résultats suggèrent que la pathogénicité de *P. tracheiphilum* peut être également modulée par les facteurs biologiques du sol.

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CONTRIBUTION OF AUTHORS

Chapter 3. Influence of soil inoculum density and environmental parameters on Pythium stunt caused by *Pythium tracheiphilum* in head lettuce crops.

This first manuscript is co-authored by the candidate, Dr. Valérie Gravel, Department of Plant Science, Macdonald campus of McGill University, and Mr. Hervé Van der Heyden (MSc.), Compagnie de recherche Phytodata inc., Sherrington. The candidate performed all the experiments, data collection, DNA extraction, PCR and statistical analysis and wrote drafts of the manuscript. Mr. Van der Heyden conceived the idea and provided funding to conduct the research. All the authors were involved in editing the manuscript.

Chapter 4. Characterization of soil suppressiveness to *Pythium tracheiphilum*

This second manuscript is co-authored by the candidate, Dr. Valérie Gravel, Department of Plant Science, Macdonald campus of McGill University, and Mr. Hervé Van der Heyden (MSc.), Compagnie de recherche Phytodata inc., Sherrington. The candidate performed the sampling, DNA extraction and PCR analysis and wrote drafts of the manuscript under the guidance of Mr. Van der Heyden and Dr. Gravel. Mr. Van der Heyden conceived the idea and provided the funding to carry out this study. Metagenomic analysis and bioinformatic processing has been performed by the Laboratoire d'Écologie Microbienne (LEM) of the Institut de Recherche et de Développement en Agroenvironnement (IRDA), under the supervision of Dr. Richard Hugues and Mr. Thomas Jeanne. Mr. Thomas Jeanne also provided useful support and advice to the candidate. All the authors were involved in the manuscript edition.

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1. General introduction

1.1 Introduction

In the muck soils of southwestern Quebec, also called organic soil or histosol, head lettuce crops are commonly infected by the soilborne pathogen *Pythium tracheiphilum*, which is responsible for Pythium stunt, a root disease that results in severe stunting and wilting of the plants. Damages resulting in yield loss are variable and, for certain period of the growing season, it can reach up to 70% of the production (Van der Heyden, H. personal communication).

Currently, estimation of the disease risk is built on historical data, and the control of *Pythium*-induced diseases in lettuce crops is mainly based on the use of two chemical fungicides, metalaxyl and cyazofamid, which are applied all over the growing season, regardless of soilborne pathogen density or meteorological conditions. The lack of knowledge concerning *Pythium tracheipilum* is certainly the main underlying cause for such fungicide utilization since no risk indicator for Pythium stunt are available to guide the producers. However, it is now well known that the repeated use of fungicide over time, especially in a context of monoculture, has led to significant issues related to soil health degradation, human health and fungicide resistance (Chellemi et al. 2016; Katan et al. 2012; Martinez et al. 2005). Therefore, there is a need for improving management of Pythium stunt in head lettuce crop to maintain the sustainability of the production.

Such a goal can be reached by the development of integrated pest management strategies (IPM), which would involve the rationalization of chemicals through an accurate knowledge of the factors influencing disease development. In plant pathology, disease

development is known to be influence by the pathogen, its host and their environment. Moreover, microbial communities inhabiting the soil, especially within the rhizosphere, have a strong influence on plant growth and health and can be involved in either the general or specific suppression of a soilborne pathogen, including *Pythium* spp. (Martin and Loper 1999). Better knowledge on both, biotic and abiotic factors, affecting the incidence of Pythium stunt in head lettuce crops is therefore essential for the development of sustainable control strategies.

The study of soilborne disease epidemic is often limited by the accuracy of identification and quantification of the pathogen in the soil, thus making the investigation of relationship between inoculum and disease difficult to achieve, especially under field conditions. However, development of molecular tools such as real-time Polymerase Chain Reaction (qPCR) has provided for a quick and specific identification and quantification of soilborne pathogen in soil samples, and several species-specific PCR primers for *Pythium* species were developed in the last decade (Klemsdal et al. 2008; Wang et al. 2003; Wang and White 1997). Moreover, in a recent study, Van der Heyden et al. (In press) reported the development of a sensitive and specific real-time qPCR assay for *Pythium tracheiphilum* and suggested that a relationship between soil inoculum concentration and Pythium stunt disease may exist.

Several examples of suppressive soil toward *Pythium* species can be found in the literature (Bruggen and Semenov 2000; Martin and Loper 1999; Raaijmakers et al. 2008), and some biocontrol agents such as *Bacillus subtilis*, *Trichoderma harzianum* or *Gliocladium catenulatum* are currently available on the market to control *Pythium*-induced

diseases. However, to the author's knowledge, nothing has been done yet relating to the possible interactions between soilborne microorganisms and *Pythium tracheiphilum*.

1.1 Research hypotheses

We expect that there is a relationship between incidence of Pythium stunt caused by *Pythium tracheiphilum* and:

1. The soil inoculum density.
2. The weather conditions. More specifically we expect that cold temperatures and high moistures will favor disease development.
3. The rhizosphere microbial biodiversity. More specifically we expect that species diversity as well as certain class of microorganisms may have a suppressive effect on *Pythium tracheiphilum*.

1.2 Objectives

The main purpose of this research was to establish a threshold of inoculum density for pathogenic *Pythium tracheiphilum* in head lettuce crops, and to identify abiotic and biotic factors fostering disease development. More specifically the objectives were:

1. To define a threshold of inoculum density under controlled conditions.
2. To establish a relationship between soil inoculum density, weather parameters (air temperature, relative humidity and rainfall) and Pythium stunt incidence under commercial field conditions.
3. To characterize microbial community of rhizosphere soil of (a) diseased and healthy plants, and of (b) lettuces grown in either a soil suppressive or conducive to *P. tracheiphilum.*

2. Literature review

2.1 Lettuce (*Lactuca sativa* L.)

2.1.1 Generality

Genus *Lactuca* are herbaceous plants with alternate leaves that include about one hundred species in the northern hemisphere. *Lactuca biennis* (Moench), *Lactuca serriola* L. and *Lactuca canadensis* L. are among the native species from Quebec and *Lactuca sativa* L. is the species from which all the cultivated varieties arise (Marie-Victorin 1935). The main types of lettuce cultivated are crisphead (iceberg), Cos (romaine), leaf (loose-leaf lettuce), butterhead and stem (asparagus lettuce) (Baysal-Gurel and Miller 2010).

2.1.2 Market

In Canada, 4 734 ha were dedicated to the production of lettuce in 2015, for a total production of 109 233 tonnes which generated over 95 479 K$. Canadian lettuce production increased by 20% between 2015 and 2014 and has doubled since 2012 (ISQ (institut de la statistique du Québec) and MAPAQ (ministère de l'Agriculture, des Pêcheries et de l'Alimentatio du Québec) 2016). In 2015, for field-grown lettuces only, 4 192 ha were dedicated to this crop in Quebec, which represent an increase of 9.6% compared to 2014. The total production in Quebec was of 90 060 tonnes in 2014 and 99 167 tonnes in 2015, generating 69 673 K$ and 83 347 K$ for 2014 and 2015 respectively. Quebec is therefore the largest producer of lettuce in Canada, as it represents 91% of the total production in Canada (ISQ and MAPAQ 2016).

2.1.3 Cultural practices

Lettuce grows well in temperate climates and in moist, rich soil with full sun exposure. Optimal pH in mineral soils is between 6.0 and 6.8 but the plant is tolerant to slightly acidic soils as well (Baysal-Gurel and Miller 2010) and for organic soil, pH of 5.4 to 5.8 is recommended (CRAAQ (centre de référence en agriculture et agroalimentaire du Québec) 2011). Lettuce is mainly produced in organic soil, also called muck soils or histosols, and can be direct-seeded or transplanted. For some growers, transplants are generally used in early spring and in August, but most of them used this method all summer long. Seedlings are grown in greenhouses in soil blocks for 3–4 weeks before transplanting. Most of the growers produce their own seedlings, but some prefer to buy them directly from specialized businesses. When direct-seeding is used, lettuces are sown in rows and thinning is required when plants reach the 4-leaf stage. Both transplanted and direct-seeded lettuces are grown on mounds in order to prevent leaf diseases that may occur from inadequate airflow. In Quebec, planting begins at the end of April, often under floating row covers, which create a warm microclimate, and harvest may last until mid-October. Lettuces are fast-growing plants that reach a marketable size within 53 days (summer) to 70 days (spring/fall) (Consortium PRISME 2016).

2.1.4 Diseases and insects

Over the growing season, lettuces are susceptible to a great range of pests. Plants can be attacked by different insects such as the tarnished plant bug (*Lygus lineolaris*), aphids (species from the Aphidoidea family), cutworms (specie from the Noctuidae family) and loopers (*Trichoplusia ni*) (Consortium PRISME 2016). Foliar diseases such as downy mildew *(Bremia lactucae*) or bacterial spot *(Pseudomonas* sp.*, Xanthomonas* sp.*)* are also major problems encountered each year in lettuce production (Consortium PRISME 2016).

However, in contrast with insects and foliar diseases, soilborne diseases are far more difficult to control and therefore represent a major constraint to lettuce production worldwide (Blancard et al. 2003). When direct-seeded, lettuces are first susceptible to damping-off caused by either *Pythium* spp., *Rhizoctonia solani* or *Fusarium oxysporum*. Over the growing period, lettuce plants are vulnerable to lettuce drop *(Sclerotinia sclerotiorum* and *S. minor)*, bottom root (*Rhizoctonia solani)*, and Pythium stunt (*Pythium tracheiphilum*) (Consortium PRISME 2016).

In lettuce crops, according to Blancard et al. (2005), several *Pythium* species may cause disease, but *Pythium tracheiphilum* is the most damaging species of all. In 1983 in the Napierville county (Québec), yield losses caused by Pythium stunt ranged between 0 and 24% (Gracia et al. 1991). Currently, yield loss due to this disease is variable, but in some cases, can reach up to 70% (Van der Heyden, H. personal communication)

2.2 *Pythium* spp.

2.2.1 Generality

The genus Pythium is part of the Oomycetes, within the clade of heterokont/chromist, in the super-kingdom of Straminipila-Alveolata-Rhizaria. Within the Oomycetes, *Pythium* spp. are found in the Peronosporales order, and in the Pythiaceae family which has basal lineage with the Peronosporacea (Levesque et al. 2010; Thines and Choi 2016).

Genus Pythium include hundreds of different species. Most of them are soilborne, inhabiting the soil, whereas others may be found in salt and fresh water environments (Gracia et al. 1991; Levesque et al. 2010). In general, they are saprobes and plant parasites,

but some are known to be pathogens of insects, algae, fishes or mammals as well (Gracia et al. 1991; Martin and Loper 1999). *Pythium* species can be generalist, like *P. aphanidermatum* and *P. ultimum,* which have a wide variety of host crops, while others are more host-specific.

Plant pathogenic *Pythium* spp. may infect their hosts at different growth stages. Some species are commonly found to infect seedlings, leading to a pre-emergence damping-off, while infection may also occur shortly after emergence, where the pathogen infects first roots and hypocotyl of seedlings, resulting in post-emergence damping-off. On mature plants, infection generally does not kill the plant but reduces plant growth since a diminished root system results in an inefficient uptake of water, nitrogen and other nutrients by the plant (Martin and Loper 1999). In some crops where roots are the harvestable product, like carrots and sugar beets, Pythium-induced diseases may lead to severe yield loss (Martin and Loper 1999). In a lesser extent, some species *(P. aphanidermatum and P. uncinulatum*) are known to cause leaf damages in lettuce production (Blancard et al. 2005). Moreover, *P. tracheiphilum* has also been observed to directly infect the above-ground part (leaf and head) of Chinese cabbage (*Brassica campestris* L.) (Moller et al. 2003).

2.2.2 Biology and ecology

All *Pythium* species are water-dependent; they need water in their environment in order to complete their life cycle (Mendoza accessed 2016). Their mode of nutrition and ecological roles are quite similar to those of true-fungi and like them they produce complex branching of mycelia (Lamour and Kamoun 2009). Soilborne *Pythium* spp. produce

different structures in order to survive in the soil (i.e.: oospores and sporangia). These structures may survive from a few days to a few years. Oospores, sporangia and, for some species, zoospores, are structures involved in the infection process, mainly through the formation of a germ tube, appresorium and haustorium able to penetrate the plant host cells (Martin and Loper 1999) (Figure 1).

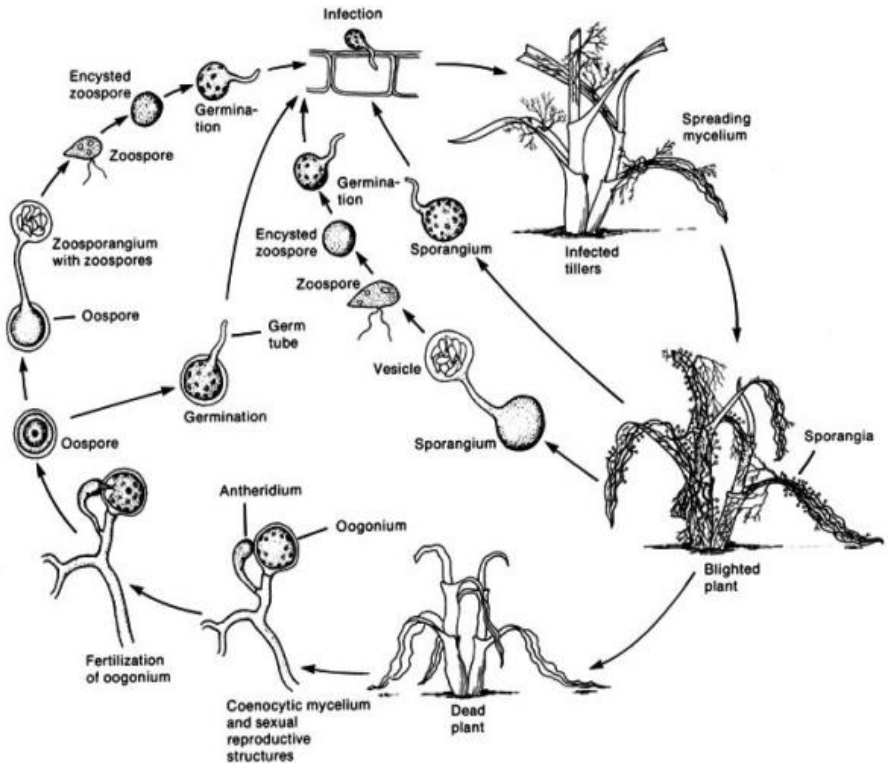

Figure 1. *Pythium* general disease cycle (Kenaga et al. 1971)

2.2.2.1 Oospores

For most of *Pythium* species, oospores, the sexual structures resulting from karyogamy (Figure 2), are known to be the main survival structure found in the soil. In the

absence of chemical stimulants, those round, double-walled, quiescent structures can survive in soils from several months to a few years. According to DeVay et al. (1982). *Pythium aphanidermatum* and *P. ultimum* are capable of surviving for 5 months in nature.

Moreover, oospores of *Pythium* spp. have been reported to be viable after 12 years in dried muck soil, being capable of infection after a 15-day wet period (Hoppe 1966). Dormant oospores germinate in response to chemical compound present in root and seed exudates, plant debris or organic matter. Chemotactic response of the oospores leads to its maturation and to the formation of a germ tube, which will penetrate host tissues and infect the plant (Martin and Loper 1999).

Oospores of *Pythium tracheiphilum* are spherical, plerotic and smooth structure with walls of 1.5 to 3.0 µm thick and a diameter of 20-25 µm (Blok and Plaats-Niterink 1978; Gracia 1989) (Figure 3a)

2.2.2.2 Sporangia

Sporangia are the second survival structure of *Pythium* species (Figure 3a). Those are asexual structures produced either by mycelium or for certain species, oospore (Figure 1) (Martin and Loper 1999). Depending on the species, it can be spherical, inflated filamentous or lobulated (Martin and Loper 1999). Sporangia are single-walled, and larger than oospore. Sporangia of *Pythium tracheiphilum* may reach 22 to 40µm when spherical and 20-45µm long and 16-29µm broad when elongated (Blok and Plaats-Niterink 1978; Gracia 1989) (Figure 3b)

Sporangia are known to stay viable over a shorter period, compared to oospores. For instance, sporangia of *P. aphanidermatum* produced in culture, showed a viability of

up to two weeks (Martin and Loper 1999; Stanghellini and Burr 1973b) while those of *P. graminicola* survived for no more than four weeks in soil (Martin and Loper 1999; Peethambaran and Singh 1977). However, sporangia of *P. ultimum* was seen to stay viable over 11 months in soil (Stanghellini and Hancock 1970). Sporangia of all species, in the presence of chemical stimulants are known to germinate directly by producing a germ tube, as oospores does.

2.2.2.3 Zoospores

In some species such as *P. tracheiphilum* and *P. aphanidermatum*, sporangia may produce motile asexual structures called zoospores (Figure 4). As described by Blok & Plaats-Niterink (1978), zoospores of *P. tracheiphilum* measure 8 to 10µm when encysted. These can move briefly in free water using their flagella. In presence of some root and seed exudates, zoospores are attracted to the root or seed surface and then attach, encyst and penetrate the host tissue through the production of a germ tube. Zoospores need to infect the host in a short time since they cannot survive desiccation. When they are encysted in the soil, they persist for as long as the soil moisture and temperature remain favorable (Martin and Loper 1999). Zoospores of *P. aphanidermatum* have been reported to survive in wet conditions for up to one week (Martin and Loper 1999; Stanghellini and Burr 1973a).

Figure 2. *Pythium* sp. oospores (Gutierrez and Melton 2001)

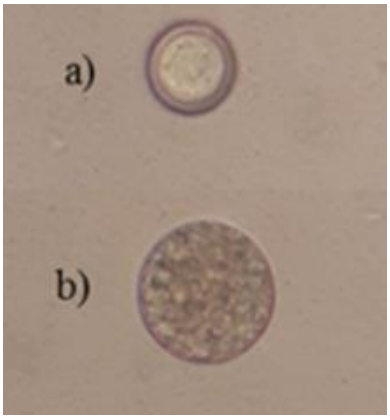

Figure 3. *Pythium tracheiphilum* structures: oospore (a) and sporangia (b) (credit photo A. Sauvageau).

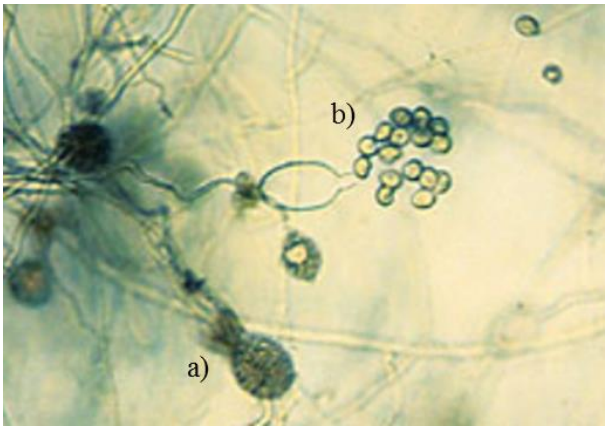

Figure 4. Pythium sporangia and zoospores. *Pythium undulatum* structures: sporangia (a) and released zoospores (b) (Courtesy P.B. Hamm from (Heffer Link et al. 2002).

2.3 *Pythium tracheiphilum* Matta

2.3.1 Distribution and hosts

Pythium tracheiphilum (Matta) is a narrow host pathogen, mostly infecting plants from the Compositea (Asteracea) family. *P tracheiphilum* has been isolated from roots of lettuce (*Lactuca sativa* L.) for the first time by Matta in 1965, in Italy (Matta 1965). Other reports of infected roots have been made a few years later in the Netherlands (Plaats-Niterink 1975) and in Germany (Blok and Plaats-Niterink 1978). More recently, infected lettuces have been reported in Norway (Nordskog et al. 2008) and in Argentina (Erlacher et al. 2015; Kiehr et al. 2015). Moreover, the species has also been reported to cause wilt and leaf blight on lettuce in Spain (Gonzalez et al. 2004) and in Australia (Kumar et al. 2007).

In muck soils of south-western Quebec, lettuces infected by *Pythium tracheiphilum* were reported for the first time in 1983 (Gracia et al. 1991). Today, in the Napierville county, *Pythium tracheiphilum* is the predominant species associated with soilborne disease in lettuce crops and it is well known to be the causal agent of Pythium stunt (Van der Heyden et al. In press).

Aside from lettuces, *Pythium tracheiphilum* has also been reported to infect *Carthamus sp.* in Alberta (Barr et al. 1997), leaf and head of Chinese cabbage in Demark (Moller et al. 2003) and Norway, endive in Spain and Australia (Berbegal et al. 2011; Kumar et al. 2007) parsnip and parsley in south eastern Australia (Petkowski et al. 2013) as well as cucumber, cauliflower and to a lesser extent tomato and pea (Blok and Plaats-Niterink 1978).

2.3.2 Pythium stunt symptoms

Pythium tracheiphilum is known to infect the vascular vessels of its host, the xylem in particular, where it spent most of its life cycle, resulting in a reduction of the plant root system and rotting of the taproot. Young plants (7 to 14 days) are generally more susceptible to infection and above-ground symptoms involves stunting and wilting of plants (Jacquet 1979). Root systems are clearly reduced while there is a great loss of secondary rootlets and undoubtedly a loss of root hairs and fine feeder rootlets as well (Figure 5). A longitudinal cut down the taproot shows a reddish-brown discoloration of the tissues (Figure 6). Even if plants may survive from infection, they usually do not reach a marketable size (Gracia 1989; Jacquet 1979).

Figure 5. Healthy head lettuce (left) and head lettuce affected by Pythium stunt (right). Both plants are the same ages (Credit photo A. Sauvageau).

Figure 6. Inside view of an infected root presenting a clear discoloration of the xylem (Credit photo A. Sauvageau).

2.4 Environmental conditions fostering *Pythium*-induced diseases

Environmental conditions affect the ecology of *Pythium spp.* in many ways. Parameters such as soil moisture, temperatures, and soil pH may have a direct effect on pathogen development but may also affect the behavior of other microorganisms inhabiting the soil, which could have an indirect effect on *Pythium* spp. diversity. Since there is not much information available for the particular species *P. tracheiphilum*, this section will combine information on other *Pythium* species in order to have a more comprehensive portrait.

2.4.1 Soil moisture

Soil moisture is the most important parameter modulating the severity of Pythium-induced diseases in agriculture and general trends are seen among the different phytopathogenic species. In most cases, high soil moisture, related to heavy rain or poor drainage, favors the development of Pythium-induced disease in crops.

Jacquet (1979), when testing different soaking time of *P. tracheiphilum* sporocysts in distilled water, found that percentage of germinated sporocysts is proportional to the time of immersion in water. The results show that germinated sporocysts reach up to 55% after a two-days soaking period. Hence, this demonstrates that water play a significant role in the early stage of germination for *P. tracheiphilum*. Similarly, under natural condition, severity of diseases caused by *P. tracheiphilum* in lettuce field seems to increase with high rainfall, overwatering and in zones of poor drainage (Gracia et al. 1991; Jacquet 1979).

Pathogenic mechanisms of *Pythium* species are modulated by soil moisture mainly because several species can produce zoospores as a means of propagation. Hence,

availability of free water becomes a key factor since it influences zoospores motility (Martin and Loper 1999). Moreover, soil moisture mediates the composition of microbial communities in the soil. It is known that, as soil moisture increases, CO_2 concentration increases whereas O_2 concentration decreases in the soil. *Pythium* species are known to be more tolerant to poor gas exchange and high CO_2 concentration than its antagonistic fungi (Martin and Loper 1999). Therefore, they are able to become predominant in high moisture soils while other types of fungi are favored as the moisture decrease (Lifshitz and Hancock 1983). Kouyeas (1964) found greater activity of *Pythium* spp. in soil at -0.4 to -1.0 bars while other soil microorganisms, such as *Penicillium* sp., *Aspergillus* sp. and *Trichoderma* sp., prevail in soil at -3.8 bars. Host susceptibility to infection has also been reported to increase under oxygen stress. For example, when tomato plants are grown under oxygen stress, the susceptibility of plants to enhanced lipoxygenase activity resulted in altered cell membranes (Cherif et al. 1997). More seeds exudation may also result from high soil moisture, which may contribute to stimulate propagule germination of *P. ultimum* (Brown and Kennedy 1966). This is consistent with results found by Jacquet (1979) who suggess that root exudate stimulates mycelial growth and sporocyst germination of *P. tracheiphilum*.

2.4.2 Soil temperature

There is an important variation in temperature responses among the different *Pythium* species. For some of them, such as *P. ultimum* and *P. aphanidermatum*, warmer temperatures seem to increase disease severity. According to Mündel et al. (1995), safflower shows less diseases caused by *P. ultimum* when grown below 15 °C compared to

greater temperatures. Similarly, more damage caused by *P. aphanidermatum* on tomato plants was seen between 27°C and 35°C (Littrell and McCarter 1970). In contrast, other species such as *P. violae* seems to prefer cooler temperature since the incidence of cavity spot of carrots caused by this pathogen was greatest at 15°C and decrease at highest temperature (Vivoda et al. 1991).

Complex microbial communities have been shown to modify temperature optima of some *Pythium* species since competition may occur at certain range of temperature. Lifshitz and Hancock (1983) found lower temperatures optima (19 to 21 °C) for *P. ultimum* in non-sterile soil compared to sterile soil (27 °C), revealing likely a competition effect at higher temperatures.

Concerning *P. tracheiphilum*, very few studies enunciate temperature optima for this species. However, when evaluating effect of different temperatures on *P. tracheiphilum* activity *in vitro*, Jacquet (1979), found that temperatures between 10 and 18°C favor its growth, multiplication and sexual reproduction, while temperatures oscillating between 18 and 24°C were optimal for sporocysts (sporangia) germination. Likewise, these results are consistent with the observation from Gracia (1978), who reported that cool temperatures occurring in spring appear to favor Pythium disease caused by *P. tracheiphilum* compared to summer months, in lettuces grown in muck soil of Quebec.

2.4.3 Soil pH

Soil pH is another parameter known to have significant influence on the pathogenic activity of *Pythium* species and they are usually found in field for which soil pH fits into a

restricted range (Martin and Loper 1999). Soil pH seems to be, among the physicochemical properties of the soil, the one that exerts the highest influence on population densities of diverse *Pythium* species (Dick and Ali-Shtayeh 1986).

For *P. ultimum*, slightly acidic soil or close to neutrality seems to be optimal for its growth. According to Alhussaen (2012), *P. ultimum* shows growth optimum between pH 6.0 and 7.0 and grows well at pH 5.0 on culture media. Under pH 4.0 and above 8.0, the pathogen only grows slightly. Similarly, when cultured *in vitro*, *P. ultimum* shows pH optimum at 5.0, but is able to grow with a pH down to 3.8 (Griffin 1958).

For *P. tracheiphilum*, similar pH optima were found in Jacquet (1979) studies. An *in-vitro* experiment showed that pH between 5 and 8 is optimal for mycelial development, whereas pH between 5.5 and 6.5 favor asexual (sporangia) and sexual (oospore) reproduction.

2.5 Control of Pythium stunt in the perspective of an Integrated Pest Management (IPM)

2.5.1 Concepts of IPM

Actual cultural practices mostly focus on increasing crop productivity. Intensive utilization of chemicals, synthetics fertilizers as well as selection for high yield cultivars in a context of monoculture has led to current issues related to soil health degradation, human health, pesticide resistance and pest control (Chellemi et al. 2016; Katan et al. 2012). These issues are now among the concerns expressed by the public, thus increasing the research for the development of new approaches in agricultural system management, more specifically pest management (Chellemi et al. 2016). Current disease management thus need to be revisited with the aim of becoming more sustainable for the years to come.

Integrated Pest Management (IPM) is a holistic approach to pest management, involving every aspect of the agricultural system, with a larger goal than that of managing one specific pest at the time (Katan et al. 2012). There is plenty of definitions for IPM (Katan et al. 2012) from these, Kogan (1998) has defined the concept of IPM in those words: " IPM is a decision support system for the selection and use of pest control tactics, singly and harmoniously coordinated in a management strategy, based on cost/benefit analyses that take into account the interests of and impacts on producers, society and the environment ". Knowledge related to pest and crop biology as well as the number of management measures are directly linked to IPM program achievement (Katan et al. 2012). These management strategies can be very diversified, including all type of soil disinfestation, biocontrol agents, organic amendments, resistant cultivars and induced resistance, cultural practices and even more and may include chemicals as a last resort (Katan 2017).

Entomologists, in the 1950s, have been first to introduce IPM concepts. Plant pathologists adopted it later, initially for foliar diseases. Management of soilborne diseases is more complicated when compared to airborne diseases (Wakeham and Pettitt 2017), therefore, its adoption for soilborne pathogens took place more recently, certainly motivated by the mandated ban of Methyl bromide following the Montreal Protocol in 1992 (Chellemi et al. 2016; Katan et al. 2012; Katan 2017). In the perspective of soilborne diseases management, Chellemi et al. (2016) as well as Katan (2017) recently discussed in a comprehensive way the pillars or principles underlying IPM of soilborne pathogens. In summary, an accurate identification of the pathogen using efficient methods as well as the prevention of its introduction and propagation in the cropping system through adequate

cultural practices are the first steps. More emphasis on the relationship between inoculum-density and disease incidence and on the factors that may influence this relationship should also be included, with the aim of developing decision support system based on the concept of thresholds. Moreover, pathogen interaction with other microorganisms should also considered: reduction of the inoculum level in soil in a way that natural biological feedback mechanisms are effective and fostering soil suppressiveness are also discussed by the authors.

2.5.2 Chemical approach and production practices

In the province of Quebec, for the control of root rot diseases or damping-off in head lettuce crop, the two sole registered chemical fungicides are metalaxyl-M (Ridomil Gold 1G, Syngenta, Guelp, Ontario, CN), and cyazofamid (e.g. Ranman 400SC and Torrent 400SC, ISK Biosciences Corporation, Concord, Ohio, USA). Both are registered for transplants grown in greenhouse that are intended to field and metalaxyl-M is also registered for soil application only for direct-seeded lettuce (CRAAQ SAgE pesticide 2017; RAP (réseau d'Avertissement Phytosanitaire) 2017). For the lettuce transplants, fungicides are applied as soil blocks drench, and the great majority of producers from the Napierville county systematically treat their transplants before transplanting in the field (Van der Heyden et al. In press).

2.5.3 Pathogen detection and identification

In the management of soilborne pathogens, as for other pathogens, confirming the pest through accurate identification is crucial for understanding etiology of the disease, becoming thus a cornerstone in the decision-making process (Lamichhane et al. 2017).

Both culture-dependant and culture-independent methods provide tools to achieve such goal, each having their proper advantage and disadvantage and hence being complementary to each other.

Culture-dependant methods are highly affordable and allow, in contrast with culture-independent methods, for the determination of " *fitness* " measurement such as sporulation, growth, pathogenicity as well as characterization of virulence and fungicide resistance. However, culture-dependant methods are time-consuming and often required expert knowledge for accurate phenotype identification (Lamichhane et al. 2017; Wakeham and Pettitt 2017). Moreover, these methods are often restricted to identification purposes as pathogen quantification remains limited (Wakeham and Pettitt 2017). On the other hand, culture-independent methods such as polymerase chains reaction (PCR) and real-time quantitative PCR (qPCR) requires shorten processing and allow to distinguish between closely related species (Almquist et al. 2016). Moreover, quantitative PCR allow measuring the concentration of target pathogens DNA, providing an interesting tool to investigate the potential relationship between soil inoculum density and disease severity (Lamichhane et al. 2017).

2.5.3.1 Culture-based approach

Simple tools such as conventional plating of plant tissue, water filtrate or soil suspensions along with observations of morphological features have long been used for isolation and identification of oomycetes including *Pythium, Phytophthora* and *Aphanomyces* species (Wakeham and Pettitt 2017). Waterhouse (1967), Plaats-Niterink (1981) and Dick (1990) provided exhaustive tools for identification of most *Pythium* species. However, since significant similarities are often seen between morphological traits

of asexual structures, distinction between *Pythium* species remains burdensome. In this context, culture-based approach for the identification of *Pythium* species remains often inadequate.

2.5.3.2 Culture-independent approach

The internal transcribed spacer (ITS) region has been widely use in mycology since this region is conserved within a species while varying between species, thus allowing for the construction of unique primer sequence (Schroeder et al. 2006; Wang et al. 2003). For identification of oomycetes, the ITS is the most commonly used region, although the cytochrome *c* oxydase region, from the mitochondrial gene, can also be an option since it has been seen to be more discriminative than ITS in some cases (Robideau et al. 2011).

Regarding the genus *Pythium,* the ITS region has been used for the study of molecular phylogeny and taxonomy of 116 *Pythium* species (Lévesque and De Cock 2004). It has also been used for the development of species-specific PCR primers allowing detection and identification of numerous *Pythium* species (Klemsdal et al. 2008; Wang et al. 2003; Wang and White 1997).

Although conventional PCR provide for a rapid and specific detection of many oomycetes species including those from the *Pythium* genus, it does not allow for quantification or direct identification from soil or plant samples (Schroeder et al. 2006). Real-time PCR, however, allows quantifying DNA by the use of fluorescent dye or probe. Numerous real-time PCR assays using the ITS region has been developed for direct quantification of soilborne pathogens in soil such as *Helminthosporium solani* (Cullen et al. 2001), *Rhizoctonia solani* AG-3 (Lees et al. 2002), *Phytophtora ramorum* (Hayden et

al. 2004) or *Colletotrichum coccodes* (Cullen et al. 2002). Species-specific primers have also been designed for detection and quantification of several pathogenic *Pythium* species in soil, for use with real-time PCR (Cullen et al. 2007; Kernaghan et al. 2008; Schroeder et al. 2006)

Recently, a real time qPCR assay using a TaqMan-MGB probe has been designed for detection and quantification of *P. tracheiphilum.* This real-time qPCR assay allows to quantify as low as 10 oospores/g dry soil, thus providing a new efficient tool for the development of integrated pest management against Pythium stunt caused by *P. tracheiphilum* (Van der Heyden et al. In press).

2.5.4 Relationship between inoculum density and disease incidence

In plant pathology, the inoculum is defined as any viable unit (i.e. cells, spores, mycelium) capable of infecting a plant and to cause disease. In this context, inoculum density refers to the density of inoculum within a soil volume or weight (Jeger and Termorshuizen 2012). Measurement of disease can be made either by looking for disease incidence or disease severity, depending on which pathosystem is studied. Disease incidence refers to a number or a proportion of diseased plants in a population, whereas the disease severity refers to as the area or proportion of symptomatic tissue on one plant (Campbell and Neher 1994). Disease severity index measured on an ordinal scale (e.g. low, medium, high severity) is also often used for measuring plant disease.

As suggested by Katan (2012), in the context of integrated pest management, the traditional routine spray programs should be replaced by a decision support system based on the relationship between inoculum density (ID) and disease incidence (DI) or severity

level. Several plant disease prediction models based on disease risk thresholds had been developed for airborne diseases combining real time weather and inoculum measurements to improve fungicide application efficacy (Gilles et al. 2004; Kleinhenz et al. 2007; Kushalappa 2001). Even though such IPM strategy are less developed for soilborne diseases, the relationship between inoculum density and disease level, combined with associated environmental parameters, can be use in a decision-support system, especially for the decisions related to crop choice, rotation or products selection (chemical or biological) (Katan 2012).

Relationship between inoculum density and disease severity or incidence has long been investigated by plant pathologists with the aim of mathematically describe soilborne diseases as a function of soil inoculum density through the fitting of linear (Boughalleb and El Mahjoub 2006; Keinath 1995; Wallenhammar et al. 2012) or non-linear models (Oyarzun et al. 1997).

More recently, the use of molecular tools such as quantitative PCR facilitated the study of the ID-DI relationship. For instance, Gangneux et al. (2014), developed a real-time PCR assay for the detection and quantification of *Aphanomyces euteiches* in soil and demonstrated positive correlation ($P<0.0001$) between inoculum potential (IP) and ID (number of oospores of *A. euteiches* per gram of dry soil).

Similarly, Almquist (2016) also developed a real-time quantitative PCR assay for *Aphanomyces cochlioides* and the relationship between pathogen inoculum density in natural infested soil and disease severity index (DS) of sugar beet root rot was assessed. Results showed a high correlation ($R^2 >0.98$) between ID and DS, thus demonstrating the

potential of inoculum density as a predictor for disease caused by pathogenic oomycete species (Almquist et al. 2016).

Several factors such as spatial and temporal variation of the inoculum distribution, virulence variation, as well as biotic interactions may vary the ID-DI relationship. To overpass the effects of such variation, the term inoculum potential has been proposed and defined by Baker (1978) as follow:

$$y = 1 - e^{-(xvfn)^m}$$

where y is the disease incidence, *m* is the slope of the ID vs the number of infections curve, and *x* (inoculum density), *y* (virulence), *f* (environmental effects), *n* (nutritional status) are factors modifying the position of the curve (Baker 1978). However, despite the relevance of each factor in the measurement of inoculum potential, they remain difficult to quantify and complexify the use of inoculum potential for a predictive model designed to reach a wider audience. Therefore, when measurement of other parameters is not affordable, inoculum density would probably be the best indicator for disease development (Jeger and Termorshuizen 2012).

2.5.5 Suppressive soil

2.5.5.1 Concept

Soils may be classified into suppressive or conductive soil, according to its ability to suppress or not the pathogenic activity of a plant pathogen. Suppressiveness of a soil is due to the combination of both, physicochemical characteristics (e.g. fertility level, drainage, soil texture, nutrients availability) and biodiversity (type and number of soil

microorganisms), which are modulating each other and result in a reduced disease incidence (Sullivan 2004). However, many discussions on soil suppressiveness have been focused on the biodiversity aspect (Bruggen and Semenov 2000; Höper et al. 1995; Martin and Loper 1999; Raaijmakers et al. 2008).

There are two known types of suppressiveness: general or specific. General suppression is due to the entire properties of the soil, which include both abiotic and biotic aspects. It owes its activity to antagonism, as well as nutrient and energy supply available to the pathogen growth. Competition for nutrients is a key factor in general soil suppressiveness. In a soil with high microbial biomass, competition for nutrient is high so less nutrient remains available for the pathogen. In contrast, in the case of high level of nutrients, the pathogen prospers (Sullivan 2004). In general suppression, microbial communities, rather than specific species, are involved and the different functional group of microorganisms found in those communities are tightly linked to different levels of decomposition of organic matter. The individual species within a functional group may change since it is the composition of functional groups, rather than individual species, that determine the character of the community (Van Bruggen and Semenov 1999)

Specific suppression, however, owes its activity to the sole effect of one or selected groups of microorganisms, such as an antibiotic producer or parasite (Weller et al. 2002). Only a few examples of specific suppressiveness are related in the literature. For instance, soilborne pathogens such as *Rhizoctonia solani* and *Scleorotium rolfsii* are not affected by the competition occurring in a general suppressive soil. For those pathogens, specific suppression involving parasitism by *Trichoderma* and *Gliocladium* can reduce the disease

potential (Sullivan 2004). However, general soil suppressiveness is more common, which is likely the case in soil suppressive to certain Pythium diseases (Boehm et al. 1997).

2.5.5.2 Microorganisms involved in suppressive soil

Microorganisms live everywhere in the soil, but it is in the rhizosphere that the highest diversity and density of microorganisms is found since root exudates are a main food resource to microbial community. Specifically, microbial populations density is estimated to be up to 20 times greater than in the bulk soil (Weller et al. 2002). These few millimeters of soil surrounding the root are therefore known to be a hot spot of microbial interaction, which will influence plant growth and health (Nihorimbere et al. 2011; Raaijmakers et al. 2008).

Genera belonging to Proteobacteria such as *Pseudomonas* (gamma-Proteobacteria) and *Burkholderia* (beta-Proteobacteria), to Firmicutes (e.g *Bacillus*), and to Ascomycota such as *Trichoderma*, *Gliocladium*, *Penicillium* and non-pathogenic *Fusarium* are well known to be part of general soil suppressiveness (Bruggen and Semenov 2000; Raaijmakers et al. 2008). In addition, some other species such as *Enterobacter cloacae* and non-pathogenic *Pythium oligandrum* and *P. nunn* have been reported to be part of suppressive soil to Pythium diseases (Martin and Loper 1999). Chen and al. (2012) also found that incidences of damping-off caused by *P. ultimum* were reduced in soil where species of *alpha- gamma-* and *beta-* Proteobacteria and Firmicutes were present (Chen et al. 2012). Similarly, Klein and al., (2013), showed that higher relative abundance of *Rhizobium* (alpha Proteobacteria), *Bacillus*, *Paenibacillus* and *Streptomyces spp.* (Actinobacteria) was found in suppressive soil to phytopathogenic *Fusarium* sp. (Klein et al. 2013).

There are typically three mechanisms by which antagonist suppress soilborne plant pathogens: competition, antagonism and parasitism. First, competition may occur for space at the root surface, for nutrients released by root and seed or for organic compounds required by the pathogen for the reactivation of survival structures and then their proliferation (Raaijmakers and al., 2009). As an example, Zhou and Paulitz (1993), found that suppression of *P. aphanidermatum*-induced root disease in cucumber, by *Pseudomonas fluorescent* and *Pseudomonas corrugate*, seemed to be related to the use of carbon and nitrogen which are required for zoospores attraction and infection. Competition for micronutrients, such as carbon and iron, has also been shown for several antagonist bacteria and fungi (Raaijmakers et al. 2008). Second, some antagonists are known to produce secondary antimicrobial metabolites, lytic enzymes or effectors that have more direct effects on the target pathogen. Certain fluorescent *Pseudomonas* as well as antagonistic fungi *Trichoderma* and *Gliocladium*, are known to produce secondary antimicrobial metabolites (Raaijmakers et al. 2008). Finally, parasitism has been well documented for *Trichoderma* and *Gliocladium*. Hyphae of these species coil around hyphae of the pathogen and enzymatic digestion follows. Some reports have been made for *P. oligandrum* showing the same type of parasitism toward *Pythium* spp. in culture (Martin and Loper 1999). Parasitism may also affect others structure, such as oospore, as it is the case for *Pythium* spp., in culture experiments (Martin and Loper 1999).

2.6 Connecting text for chapter 3

The following chapter will be submitted to an international journal for publication in a near future. The following study was carried out in line with previous works conducted by the Compagnie de recherche Phytodata inc., where the identification of *Pythium tracheiphilum* as the main causal agent of Pythium stunt in head lettuce production of the Napierville county was determined and specific molecular marker has been developed in order to facilitate research concerning Pythium-induced disease in this particular region. In the following chapter, the authors intend to determine the relationship between Pythium stunt incidence, soil inoculum density of *P. tracheiphilum* and environmental parameters such as temperature and rainfall.

3. Influence of soil inoculum density and environmental parameters on Pythium stunt caused by *Pythium tracheiphilum* in head lettuce crops.

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Keywords: Pythium stunt, Soilborne disease, Inoculum density, qPCR assay

3.1 Abstract

In Québec muck soils, Pythium stunt (*Pythium tracheiphilum* Matta) is responsible for important yield losses in head lettuce crops each year, which can reach up to 70% in certain cases. Despite the significance of the disease, factors influencing its development remain poorly documented and no disease risk indicators are available to guide producers, making disease control management difficult. Hence, head lettuce growers systematically resort to the use of chemical fungicides, which are applied all over the growing season. It is known that soilborne diseases incidence or severity may be influenced by soil inoculum density and environmental parameters. Therefore, the objectives of the present study were to investigate the influence of inoculum density on lettuce growth under controlled conditions, and to evaluate the influence of soil inoculum density, air temperature, relative humidity and rainfall on the disease incidence under field conditions, with the aim of determining accurate predictors for Pythium stunt incidence. Results show that, under controlled environment, a threshold of inoculum density of 97 spores/g dry soil (cv. Estival) and 46 spores/g dry soil (cv. Prestige) was needed to reduce lettuce dry weight by

half. Under field conditions, a percentage of disease incidence of 5% or more can be predicted by air temperatures below 18°C during the first two weeks after lettuce planting, a rain accumulation above 64 mm for the first three weeks, and a soil inoculum density above 132 spores/g dry soil. Combination of the three predictors was found to accurately predict the disease incidence 79% of the time.

3.2 Introduction

In Canada, more than 90% of the lettuce (*Lactuca sativa* L.) production is grown in the province of Quebec and most of this production is conducted in muck soils (histosols) located in the Napierville County. Pythium stunt caused by the soilborne pathogenic species *Pythium tracheiphilum* (Matta) has been reported in lettuce crops for the first time in Quebec in 1983 and resulted in up to 24% of yield loss (Gracia et al. 1991). More recently, *P. tracheiphilum* was identified to be the predominant species associated with Pythium-induced diseases in head lettuce crops and related yield losses have increased to reach up to 70% (Van der Heyden, H. personal communication). *Pythium tracheiphilum* is known to infect vascular vessels of its host, the xylem in particular, where it spends most of its life cycle, resulting in root rot diseases. Young plants (7 to 14 days) are generally more susceptible to infection and above-ground symptoms involves stunting and wilting of lettuce plants (Jacquet 1979). The root system is clearly reduced while there is a great loss of secondary roots, root hairs and fine feeder rootlets. A longitudinal cut down the taproot shows a reddish-brown discoloration of the tissues. Even if plants may survive from infection, they usually do not reach a marketable size (Gracia 1989; Gracia et al. 1991; Jacquet 1979). *Pythium tracheiphilum*, as other oomycetes, produces oospores that could remain latent in soil from several months to years. Sporangia is another structure capable

of infection, however, it is known to stay viable for a shorter period compared to oospores (Martin and Loper 1999). As Quebec's growing seasons are interrupted by a 6-month cold period, it is likely that pathogens structures recovered from the soil in the spring would be long-term survival structures, mostly oospores. Accordingly, the concentration of spores (oospore and/or sporangia) found in soil before the first crop could be a good indicator of subsequent Pythium stunt disease severity in head lettuce crops. Moreover, in a recent study, Van der Heyden et al. (In press) reported the development of a sensitive real-time qPCR assay and suggested that a relationship between soil inoculum concentration and *P. tracheiphilum* root rot disease may exist.

When evaluating effect of temperature and moisture on the development of *P. tracheiphilum* growing on culture media, Jacquet (1979) showed that temperatures between 10 and 18°C favor its growth, multiplication and sexual reproduction. Similarly, temperatures oscillating between 18 and 24°C as well as a 36-hour period under water was optimal for the sporocysts germination. Under field conditions, severity of diseases caused by *P. tracheiphilum* in lettuce field seemed to increase with high rainfall, overwatering and in zones of poor drainage (Gracia et al. 1991; Jacquet 1979).

Despite the significance of *P. tracheiphilum*-induced disease in Quebec and elsewhere, factors influencing the disease development, such as soil inoculum and environmental conditions, remain poorly documented making disease control management difficult. Hence, head lettuce growers of Napierville county systematically resort to the use of chemical fungicides (cyazofamid or metalaxyl) all over the growing season, which results in monetary and environmental costs. In fact, such repeated use of certain pesticides over time is also known to reduce the diversity of soil life and to favor pathogen resistance

(Sullivan 2004). For instance, Martinez et al. (2005) studied the efficiency of different fungicide to control cavity spot of carrot (*Pythium sulcatum, P. sylvaticum* or *P. macrosporum*) and found that a repeated use of zoxamide, the only efficient fungicide in their study, resulted in a loss of sensitivity for specific *Pythium* isolates.

The first step to rationalize pest control management is to identify the conditions under which the use of chemicals is necessary from those when it is possible to avoid, diminish or replace their utilization. Therefore, the aim of the present study is to characterize biotic (*P. tracheiphilum* inoculum density) and abiotic factors (temperature, relative humidity and rainfall) influencing the development of Pythium stunt in head lettuce production. Specifically, the objectives of the study were to i) determine the relationship between soilborne inoculum and Pythium stunt under controlled conditions, ii) examine the relationship between soil inoculum density and disease incidence under field conditions and iii) evaluate the influence of environmental parameters on disease incidence under field conditions.

3.3 Materials and methods

3.3.1 Controlled conditions experiments

3.3.1.1 Pathogen isolation

Symptomatic lettuce plants were collected from commercial fields in Napierville county (app. 45°08'37"N and 73°34'22"W) during the growing seasons of 2014, 2015 and 2016. Whole plants were first washed under running tap water. Pieces of roots were cut and soaked for 1 min in distilled water, 1 min in 1% sodium hypochlorite, and 1 min in distilled water. Infected root tissues were selected and cut to 1–2 mm length and placed on

the surface of potato dextrose agar plate (PDA, Becton, Dickinson and Company, USA). Pure cultures were obtained by transferring disc covered with hyphal tips on PDA, and identification of *P. tracheiphilum* was made by sanger sequencing (ITS1 and ITS4) (White et al. 1990). Pure cultures were stored in sterile water and placed at room temperature until use.

3.3.1.2 Preparation of *P. tracheiphilum* inoculum and inoculation procedures

A combination of different strains of *P. tracheiphilum*, previously isolated from symptomatic field-grown lettuces, were used in each experiment. Plugs from PDA colonies were plated on 90 mm petri dish containing V8 juice agar medium (200 ml of V8 juice, 3g of $CaCO_3$, 15g of agar and tap water to complete 1 liter) and placed at room temperature for 14 days to induce sporangia and oospores formation. Inoculum was prepared by soaking the culture with 7ml of distilled water. Then, the agar surface was gently scraped with a scalpel blade to recover mycelium, oospores and sporangia. The mixture was first agitated and then filtered through cheesecloth to remove mycelial structures. An approximate amount of 100ml of water was used to wash the cheesecloth in order to recover as many spores as possible. Concentration of the stock solution obtained was quantified using a hemacytometer to provide a number of spores/ml. Appropriate aliquot were taken from the stock solution and added to 100ml of water in order to obtain an inoculum density expressed in number of spores per gram of dry soil. Different inoculum densities used were 0, 10, 25, 50, 100, 500, 1000 or 5000 spores/g of dry soil. Each solution was added to soil and mixed before transplanting. The soil used was a commercial soil composed of manure, peat and loam (Mix 3, Les sols Isabelle Inc., St-Michel, Qc, CN), and was previously

partially sterilized by placing it in an oven at 90°C for 30 min, twice, to reduce the possible effect of biological activities within the soil.

Two different cultivars of lettuce (*Lactuca sativa* L.) were tested in two different sets of experiments: cv. Prestige, a susceptible cultivar, and cv. Estival, a cultivar that is used by most of the lettuce producers from the Napierville county. Two trials of the same experiment were conducted for each cultivar. Two-week-old lettuce plants were transplanted in individual 4-inch square plastic pots containing inoculated soil, one plant per pot. Each pot was placed in a plastic bag to avoid cross-contamination between the different inoculum concentrations. Lettuce plants were grown in a growth chamber for two weeks under temperature and air relative humidity set to 21°C ± 3°C (day) and 15°C ± 3°C (night), and to 70% ± 5%, respectively. Details on the measured environmental conditions in the growth chamber are given in table 1.

Photoperiod was adjusted to 14h with a light intensity of 1280 µmol/m2/s. Lettuces were individually watered with 100 ml the first day, followed by 50-75 ml every two days. Experimental design was a randomized complete block design with 6 blocks and 8 inoculum densities per block. After two weeks, whole plants were harvested and carefully washed under running tap water. Plants were then dried in an oven at 60°C and dry weight was measured.

3.3.1.3 Validation

In order to confirm that *P. tracheiphilum* was the causal agent of the observed symptoms, root pieces, previously washed under running tap water and then soaked for 1min in distilled water, 1 min in 1% sodium hypochlorite, and 1 min in distilled water, were

placed on water-agar (WA) medium for 2 days at ambient temperature and then on PDA medium. Validation was made by visual identification of the mycelial growth, first on water-agar, and then on PDA. Two plants per treatment were used for the validation. To maintain the virulence of the strains used, PDA cultures obtained from the first experiment were used to inoculate the V8 medium used in the next experiment.

3.3.1.4 Statistical analysis

First, the assumption of normal distribution was tested using the Kolmogorov-Smirnov goodness-of-fit tests (SAS Proc Univariate, SAS institute). Two-way ANOVA model with three factors were conducted using SAS Proc GLM software. Inoculum densities were specified as treatment factor, blocking was specified as Block factor and the experimental replicates (trial 1 and 2) was specified as Time factor. A least significant difference (LSD) test was performed when there was a treatment effect, to distinguish which inoculum density differed. Plant dry weight (*y*) was described by the following sigmoidal dose-response function:

$$y = min + \frac{max - min}{1 + 10^{(logEC50 - x)}}$$

where *x* is the inoculum concentration (spores per gram of dry soil on a ln scale), *min* is the minimum response, *max* is the maximum response and EC_{50} is the concentration providing a response halfway between the minimum value and the asymptote. Adjusted R^2, parameters and equation for sigmoidal dose-response curve were fitted using Sigma plot (Systat Software Inc.). Inoculum density threshold was set according to the EC_{50} value obtained.

3.3.2 In-field experiment

3.3.2.1 Data collection

From May to July 2016 and 2017, a total of 134 experimental plots were established in 20 fields, allocated over 6 lettuce growers in the Napierville county (Table 2). Each experimental plot was a quadrat of 5 meters by 5 meters, containing 192 lettuce plants. Before cropping, soil from each plot was sampled, placed in plastic bags and frozen at -20°C until use. Within each experimental plot, soil samples were made from 15 random soil subsamples taken vertically from the first 15 centimeters of soil using an auger.

In 2016 and 2017, number of symptomatic plants (wilted or stunted plants presenting yellow-brown discoloration of the root xylem) within each plot was counted twice during the growing season (about 45 days); the first time when lettuce plants were at the 14 leaf-stage and a second time when the head had reached a diameter of 10 cm. Diseased plants were removed from the plot during each evaluation to avoid recounting during the next evaluation. Sum of all evaluations were used to calculate the final percentage of disease incidence ((number of diseased plants / total number of plants) *100). The total number of plants refers to the total number of lettuces planted within the experimental plot before evaluation.

Air temperature (°C), relative humidity (%), and rainfall (mm) were collected from weather stations (Watchdog 2700, Spectrum Technologies, Inc., IL, USA) placed outside the field at a maximum distance of 5 kilometers. Weather parameters were monitored every hour. In 2016, soil temperature at 10 cm (°C) and soil humidity at 10 cm (% Volumetric Water Content) were also collected in certain fields using the same weather stations.

Measurement of the soil pH for each sample was also made using a pH meter (pH150 Meter, OAKTON instruments, IL, USA).

3.3.2.2 DNA extraction and qPCR analysis

DNA extraction from soil samples was made on 0.20 g of dry soil using the FastDNA SPIN kit for soil (MP Biomedicals, Solon, USA), following the manufacturer instructions. Briefly, 0.20 g of soil and 1.1 ml of Buffer was added to lysing matrix tube, homogenized and centrifuged, and then protein precipitation was made by adding 250 µl of PPS and centrifuged again. Binding Matrix (800 µl) was added to the remaining supernatant solution. Purification step was done using 500 µl of SEWS-M and final DNA elution was made in 100 µl of DES. Quantity and purity of the collected DNA was measured by spectrophotometry using Nanodrop lite (Thermo Scientific, Massachusetts, USA).

Real-time Taqman qPCR protocol described by Van der Heyden (In press) was been used for the detection and quantification of *P. tracheiphilum* in soil. A CFX connect real time qPCR instrument (Biorad, Mississauga, On, Canada) was used for the qPCR assay. Each reaction (25 µl) contained 300 nM of each primer (909F, PTMGB-R, EIPIC100F and EIPC100R), 100 nM of each probe (PTMGBP and EIPC100P), 0.06 ng of BSA, 6 mm of $MgCl_2$, 1X PerfeCTa qPCR ThoughMix (Quanta Bioscience, Beverley, MA) and 3 µl of gDNA. Cycling conditions were set to 95°C for 5 min, 40 cycles at 95°C for 15 s, and 62°C for 20 s. To serve as a reference to measure PCR inhibition, a negative control with only the internal control was included in each qPCR run (Van der Heyden et al. In press).

3.3.2.3 Correlation analysis

Correlation between disease incidence (DI) and soilborne inoculum density (ID), air temperature, relative humidity, and rain accumulation was assessed using a Spearman's rank correlation matrix (Proc Corr., SAS 9.4). Because each experimental plot had their specific transplanting date, the average of daily air temperature during the first week (ATW1), the first two weeks (ATW12) and the first three weeks (ATW123) after transplanting in the field was calculated for each experimental plot. The same thing was done for the air relative humidity (RHW1, RHW12 and RHW123) and precipitation (RW1, RW12 and RW123) weeks after transplanting. For 2016, soil temperature (STW1, STW12, STW123), soil humidity (SHW1, SHW12, SHW123) and soil pH (SpH) were also measured for the same three periods and correlated with disease incidence using a Spearman's rank correlation analysis. Soil inoculum density was ln-transformed (ln(ID+1) before the correlation test (Standard Procedure, SAS 9.4).

3.3.2.4 Determination of conditions fostering disease incidence

Based on field data collected in 2016 and 2017, experimental plots were divided into two groups: those with a disease incidence of less than 5% was described as healthy (0), and those with more than 5% were described as diseased (1). Based on the most correlated parameters (Table 6), a binary recursive partitioning (BRP) analysis (JMP 13 Pro, SAS institute) was used to build a decision tree. Briefly, in a BRP analysis, the algorithm predicts a response variable by successively partitioning (splitting) the explanatory variables into two subgroups (a node is split into a right and left branch) (Merkle and Shaffer 2011), where the optimal cut point for a split is the one which

maximise the LogWorth (LogWorth statistic is $-\log_{10}$(p-value)) (SAS Institute Inc. 2017.). Therefore, this type of analysis allows to set threshold values (cut point in each split) that will distinguish the " healthy " group from the " diseased " group. In the creation of a decision tree involving a categorical response (as in the present study) the variable chosen at each split is the one that maximise the likelihood-ratio chi-square (G^2) (SAS Institute Inc. 2017.). Notice that in JMP, before being selected for a split, the explanatory variables are named "candidate". In this study, the minimum size split was set to 10, which means that the node was not partitioned if there were less than 10 observations (count) in each branch.

In a BRP analysis, to validate the predictive model, a K-fold Cross-validation can be apply, which allow to compare the predictive accuracy of a "training" dataset (the complete dataset) with a "testing" dataset (k subgroups of the complete dataset) (SAS Institute Inc. 2017.). Cross-validation is also useful when the optimal number of split is to be determined. However, in the present study, the number of split was predetermined to be at least 3, as each of the three parameters (ID, ATW12 and RW123) needed to be assessed in the partition model and needed to be involved only one time, in order to get a more simplify decision tree. Therefore, the K-fold Cross-validation was not computed in this study. Notice that the use or the non-use of the K-fold Cross-validation procedure will not affect the cut point values or the choice of the candidate at each split, it will only affect the optimal number of split to retain and its related R^2.

In BRP analysis, no significance tests are involved, therefore, the predictive accuracy is used to evaluate the 'goodness of fit' of the tree (Merkle and Shaffer 2011). Accordingly, a contingency table was calculated in order to assess the reliability of using

the thresholds found with the BRP analysis for the prediction of the disease incidence. Seven combinations of predictive parameters were included in the contingency table. First, each predictive parameter was assessed alone, then all combinations of two, and the combination of the three parameters. Each experimental plot with disease incidence of 5% or more was classified as observed (O+), and those with disease incidence lower than 5% were classified as not observed (O-). The disease was predicted (P+) when the predictive parameters (DI, ATW12, RW123) were above their respective threshold, while the disease was not predicted (P-) when one or more predictive parameters were above their respective threshold. Hence, four different cases are possible. First, a " true positive " (TP) is noted when the disease was predicted (P+) and observed (O+). Second, a " true negative " (TN) is noted when the disease was not predicted (P-) and not observed (O-). Third, a " false positive " (FP) is noted when the disease was predicted (P+) but not observed (O-). And fourth, a " false negative " (FN) is noted when the disease was not predicted (P-) but observed (O+). The " *sensitivity* " of the test was designated by the true positive proportion (TPP): [TP/(TP+FN)]. The " *specificity* " of the test was designated by the true negative proportion (TNP): [TN/(TN+FP)]. The proportion of correct assessment [TP+TN / (TP + TN + FP + FN)] was calculated to provide the " *overall accuracy* " of the test (Fall et al. 2015).

Table 1. Measured conditions in the growth chamber.

			Air temperature (°C)		Soil temperature[d] (°C)		Relative humidity (%)	
			Average[a]	Variance	Average	Variance	Average	Variance
cv. Estival	Trial 1	Day[b]	21.1	0.8	16.8	0.6	46.6	14.0
		Night[c]	16.3	1.1	15.4	0.6	65.7	31.8
	Trial 2	Day	20.1	0.3	16.6	0.8	53.4	24.6
		Night	16.0	1.1	14.6	0.5	63.3	38.8
cv. Prestige	Trial 1	Day	21.2	0.9	18.0	4.0	48.5	13.5
		Night	16.0	1.6	15.8	1.6	81.2	33.3
	Trial 2	Day	20.8	1.5	17.3	6.1	51.4	103.6
		Night	15.9	2.0	15.0	4.3	81.5	68.9

[a]Average of 14 days

[b]Refers to 14 hours of lighting

[c]Refers to 10 hours in darkness

[d]Soil temperature was taken in the middle of one pot (depth of about 5cm) that was placed in the middle of the growth chamber

Table 2. Latitude and longitude coordinates related to each field sampled in the study.

Farm Code	Field	Latitude	Longitude
A	G1	45°10'06.3"N	73°37'14.9"W
B	B1	45°08'00.7"N	73°36'05.6"W
	B5	45°07'53.2"N	73°36'04.7"W
	VN14	45°07'08.8"N	73°31'43.4"W
	VN301-308	45°08'40.1"N	73°29'57.7"W
	VN31	45°08'07.8"N	73°31'35.8"W
	VN32	45°08'14.6"N	73°31'29.5"W
	VERN240	45°08'44.3"N	73°29'13.2"W
	1.3	45°09'15.7"N	73°37'32.9"W
	2.4	45°09'04.9"N	73°37'53.0"W
C	Lab2A	45°09'06.9"N	73°39'02.9"W
D	MOQ3-5-6	45°09'05.2"N	73°27'09.1"W
	A3C3	45°07'55.8"N	73°27'23.3"W
	BAB	45°07'51.8"N	73°26'14.3"W
E	24	45°07'34.5"N	73°31'51.5"W
	27	45°07'23.8"N	73°32'01.5"W
	31	45°07'44.1"N	73°31'57.6"W
	35	45°07'28.1"N	73°32'17.7"W
	5	45°07'27.4"N	73°31'05.3"W
	51	45°08'36.1"N	73°33'47.6"W

3.4 Results

3.4.1 Controlled conditions experiments

For growth chamber experiments involving the cultivar Prestige, there is a significant effect of inoculum density (p <0.0001) but no Block effect (p =0.8843) on dry weight. However, since time effect was significant (p >0.001) (Table 3) the LSD test has been performed on each individual trial. In the first trial, the dry weight of lettuces grown in 10 spores/g dry soil was not significantly different from the non-inoculated controls (Table 3). However, plants grown at higher concentration shows significant differences. Soil inoculum density of 25 spores/g dry soil was significantly different from 50, 500 or 1000 spores/g dry soil, and the highest concentration of 5000 was significantly lower than all other concentrations. Concentration of 100 spores/g dry soil, however, was not different from 25, or from 50, 500 and 1000 spores/g dry soil. In the second trial, the dry weight of lettuces grown with a soil inoculum density of 10, 25 or 50 spores/g dry soil was not significantly different from the non-inoculated controls, but weight decreased at higher concentration. Densities of 100, 500 and 5000 spores/g dry soil were not significantly different from each other. The dry weight of lettuces grown with 1000 spores/g dry soil was unexpectedly high, and was not different from concentration 25, 50, 100 or 500 spores/g dry soil (Table 3).

For growth chamber experiments involving the cultivar Estival, the ANOVA analysis shows a significant effect of inoculum density (p <0.0001) and Block effect (p =0.0002) on the dry weight. Time effect was not significant (p = 0.9676) confirming the reproducibility of the results (Table 3). The LSD test was conducted to assess any

significant difference between each inoculum concentration, in terms of dry weight. For this test, a combination of both trials was used. Lettuces grown at inoculum density of 10, 25 or 50 spores/g dry soil were not significantly different in their dry weight when compared to the non-inoculated controls (Table 3). However, plants grown at higher concentration had lower dry weights, but a linear decrease was not seen, as concentration of 100, 500 and 1000 spores/g dry soil were not significantly different from each other. The highest concentration of 5000 was not different from 500 or 1000 but was different from all other concentrations (Table 3).

For both cultivars taken separately, dry weight (y) was described by a sigmoidal dose-response function (Figures 7 and 8). Coefficient, standard error, t and p value for equation parameters as well as adjusted R^2 are shown in table 4. For the first trial with cultivar Prestige, coefficient of determination was slightly lower ($R^2_{adj} = 0.692$) when compared to the second trial ($R^2_{adj.}$ of 0.726). Values for the EC_{50} corresponded to 26 spores/g of dry soil and 61 spores/g dry soil for trial 1 and trial 2, respectively (Table 4). For cultivar Estival, sigmoidal dose-response function suggested an EC_{50} corresponding to 97 spores/g of dry soil ($R^2_{adj.}$ of 0.936) (Table 4).

Table 3. Results of the ANOVA and LSD analysis conducted for controlled experiments involving the lettuce cultivars Prestige and Estival.

		cv. Prestige			cv. Estival
		Trial 1	Trial 2	Trials 1&2	Trials 1&2
ANOVA					
	Model	<.0001	0.0007	<.0001	<.0001
	treatment	<.0001	<.0001	<.0001	<.0001
	Block	0.998	0.8263	0.8843	0.0002
	Time	…	…	<.0001	0.9676
	treatment*Time	…	…	0.1091	0.839
LSD					
	0[a]	0.37[b] a	0.44 a	…	0.39 a
	10	0.35 a	0.42 a	…	0.37 a
	25	0.30 b	0.36 abc	…	0.35 a
	50	0.24 c	0.39 ab	…	0.33 a
	100	0.28 bc	0.27 cde	…	0.3 b
	500	0.25 c	0.24 de	…	0.27 bc
	1000	0.24 c	0.32 cdb	…	0.27 bc
	5000	0.17 d	0.2 e	…	0.25 c

[a] Inoculum densities expressed in number of spores per gram of dry soil

[b] Lettuce dry weight expressed in gram. Mean of 6 replicates.

Table 4. Coefficient, standard error and adjusted coefficient of determination (R^2adj) for sigmoidal dose-response function describing the relation between *Pythium. tracheiphilum* soil inoculum density and lettuce dry weight.

Cultivar	Trial	Parameters	Coefficient	Standard error	t	*P*	Spores/g dry soil[a]	$R^2_{adj.}$
Prestige	1							0.692
		min	0.363	0.030	12.261	<0.0001	…	
		max	0.228	0.018	12.614	<0.0001	…	
		logEC50	3.292	0.482	6.827	0.001	26	
	2							0.726
		min	0.425	0.031	13.733	<0.0001	…	
		max	0.230	0.026	9.484	0.0002	…	
		logEc50	4.092	0.459	8.921	0.0003	61	
Estival	1 & 2							0.936
		min	0.492	0.011	44.717	<0.0001	…	
		max	0.269	0.011	24.553	<0.0001	…	
		logEC50	4.587	0.150	30.569	<0.0001	97	

[a]Equivalent of the EC50 value in number of spores per gram of dry soil.

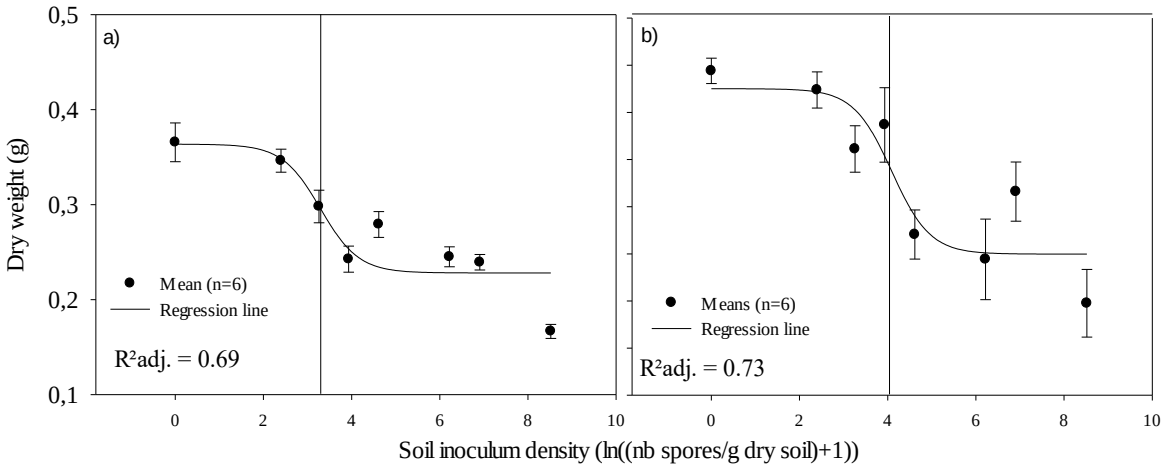

Figure 7. Relationship between *Pythium tracheiphilum* soil inoculum density and lettuce (cv. Prestige) dry weight for trial 1 (a) and trial 2 (b). Points are the means of 6 replicates. Bars are standard errors of the mean. Regression used the mean of the 6 replicates per inoculum density. Vertical line is placed at the EC50 value.

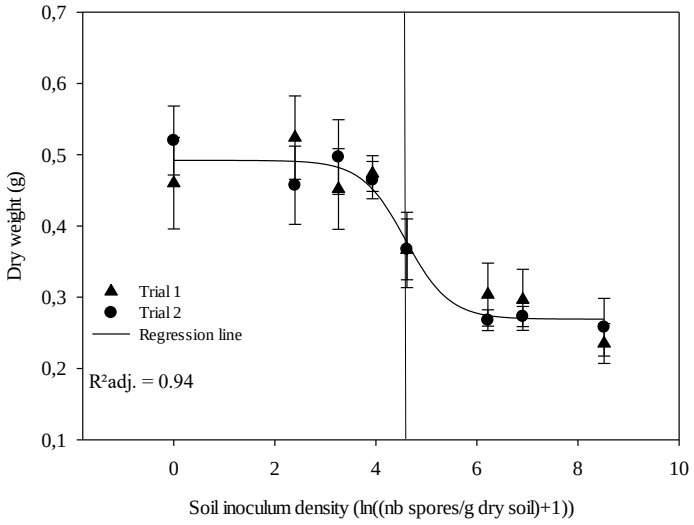

Figure 8. Relation between *Pythium tracheiphilum* soil inoculum density and lettuce (cv. Estival) dry weight. Points are the mean of 6 replicates. Bars are standard errors of the mean. Regression used the average of trial 1 and trial 2, n = 12 for each inoculum density. Vertical line is placed at the EC50 value.

3.4.2 Field trial

In 2016, the disease incidence (DI) mean ranged from 0 to 5.9%, with an average of 2% over the 79 experimental plots evaluated. Soilborne inoculum density (ID) ranged from 0 to 521 spores/g dry soil with an average of 141 spores/g dry soil. In 2017, DI ranged from 0 to 8.5% with an average of 2.5% over the 55 experimental plots evaluated, and ID ranged from 0 to 4997.9 spores/g dry soil, with an average of 1199 spores/g dry soil (Table 5).

In 2016 and 2017, disease incidence was positively correlated with soilborne inoculum density (r = 0.46 and p = <0.0001 in 2016; r = 0.32 and p = 0.016 in 2017; r = 0.44 and p = <0.0001 in 2016-17). In 2016, air temperature occurring in the first two weeks after transplantation was more strongly negatively correlated with DI (ATW12 of 0.58, p = <0.0001) than air temperature during the first or the first three weeks. In 2017, air temperature for the first two and the first three weeks were similarly correlated and were more correlated than air temperature for the first week (Table 6).

Accumulation of rain was positively correlated with DI in both years, accumulation during the first two weeks had a better correlation in 2016 than the other periods (RW12 is of 0.32, p = 0.0038), and accumulation during the first three weeks had a better correlation in 2017 (RW123 is of 0.59, p = <0.0001), the two other periods being not significant (Table 6).

In 2016, the air relative humidity for the first three weeks was the only period correlated with DI (RHW123 is of -0.37, p = 0.0009). In 2017, the first and the two first

weeks were negatively correlated with DI (RHW1 is of -0.48, p = 0.0002; RHW12 is of -0.30, p = 0.0249) but the first three weeks was not correlated (Table 6).

In 2016, soil temperature was negatively correlated with the disease incidence for all three periods. However, soil temperature for the first three weeks (STW123) was more correlated (-0.39, p = 0.001) than for the first two (-0.37, p = 0.015) weeks (Table 6). Soil humidity (SH) was more correlated for the first week (0.32, p = 0.038) than for the first two (0.30, p = 0.048) weeks after the planting (Table 6). Soil pH was not correlated with disease incidence (Table 6).

Overall for summer 2016 and 2017 combined, ATW12 was the parameter most strongly correlated with DI (-0.51, p = <0.0001), followed by ID (0.44, p = <0.0001) and RW123 (0.36, p = <0.0001) (Table 6).

Table 5. Soil inoculum density and disease incidence for each field evaluated in the study.

				Inoculum density[a]		Disease incidence[b]	
Year	Farm Code	Field name	N[c]	Mean	σ[d]	Mean	σ
2016	A	G1	4	42.2	40.5	4.6	5.2
	B	B1	6	111.3	64.3	0.2	0.3
		B5	3	39.8	49.5	0.0	0.0
		VN14	2	0.0	0.0	1.3	0.4
		VN301	3	0.0	0.0	0.0	0.0
		VN308	3	7.7	8.2	0.0	0.0
		VN31	1	19.2	-	0.0	-
		VN32	2	26.5	24.3	1.3	1.8
	C	BAB	6	329.2	221.3	1.3	0.8
		MOQ3	3	79.8	78.7	1.2	2.1
		MOQ5	3	112.7	102.8	4.0	2.9
		MOQ6	3	521.1	430.3	5.4	3.3
	D	24	3	178.9	45.9	2.7	1.7
		27	8	127.5	77.1	3.2	4.0
		31	3	25.9	5.2	1.2	1.3
		35	23	340.7	182.1	5.9	5.7
		5	3	436.4	230.3	1.9	1.1
2017	B	1.3	7	482.5	174.9	3.9	2.4
		2.4	14	309.8	240.1	1.9	2.1
		VERN240	4	0.0	0.0	0.3	0.5
	E	Lab2A	3	471.2	454.3	0.0	0.0
	C	A3C3	5	4997.9	7678.4	0.3	0.5
	D	24	11	979.9	423.3	8.5	3.7
		51	11	1153.9	564.0	2.9	2.4

[a]Expressed in spores/g dry soil o

[b]Percentage of diseased plants within the experimental plot

[c]Number of experimental plots

[d]Standard deviation of the mean

Table 6. Correlation between disease incidence (DI) and weather parameters.

	2016		2017		2016-17	
Parameters[a]	*r* [b]	n	*r*	n	*r*	n
ID[c]	0.46**	79	0.32*	55	0.44**	134
ATW1[d]	-0.32**	79	-0.24	55	-0.31**	134
ATW12	-0.58**	79	-0.47**	55	-0.51**	134
ATW123	-0.39**	79	-0.47**	55	-0.42**	134
RW1[e]	0.30**	79	-0.17	55	0.18*	134
RW12	0.32**	79	0.16	55	0.28**	134
RW123	0.20	79	0.59**	55	0.36**	134
RHW1[f]	0.23	79	-0.48**	55	-0.07	134
RHW12	0.02	79	-0.30*	55	-0.07	134
RHW123	-0.37**	79	-0.26	55	-0.34**	134
STW1[g]	-0.18	43	...	...	...	...
STW12	-0.37*	43	...	...	...	...
STW123	-0.39**	43	...	...	...	...
SHW1[h]	0.32*	43	...	...	...	...
SHW12	0.30*	43	...	...	...	...
SHW123	0.16*	43	...	...	...	...
SpH[i]	0.03	62	...	...	...	...

[a] Where W1 referred to the first week, W12 to the first two weeks and W123 to the first three weeks after lettuce planting

[b] Spearman's rank correlation coefficient r

[c] Inoculum Density of *Pythium tracheiphilum* in soil. ID is expressed in ln ((nb spores/ g dry soil) +1)

[d] Average daily air temperature (°C)

[e] Rain accumulation (mm)

[f] Average daily air relative humidity (%)

[g] Average daily soil temperature (°C)

[h] Average daily soil humidity (%VWC)

[i] Soil pH

* Significant at the 0.05 level

** Significant at the 0.01 level

Based on the most correlated parameters (ATW12, ID and RW123), a binary recursive partitioning (BRP) analysis was used to create a decision tree (Figure 9). The "diseased" subgroup is represented in the left branches of the tree while the "healthy" subgroup is in the right branches.

For the first split, the selected candidate was ATW12 (G^2 is of 34.6) and the cut point value was set to 18.2°C (Table 7). The G^2 related to the "disease" subgroup for this first split is of 0, reflecting the fact that no disease cases were found above 18°C (Figure 9). For the second split, the selected candidate was RW123 (G^2 is of 18.3) and the cut point was set to 64.3 mm (Table 7). Finally, for the third split, the selected candidate was ID (G^2 is of 6.0) and the cut point was set to 4.88 (spores/g dry soil on a ln+1 scale), which correspond to 132 spores/g dry soil. As all three parameters were selected in the first three splits, no additional split was necessary.

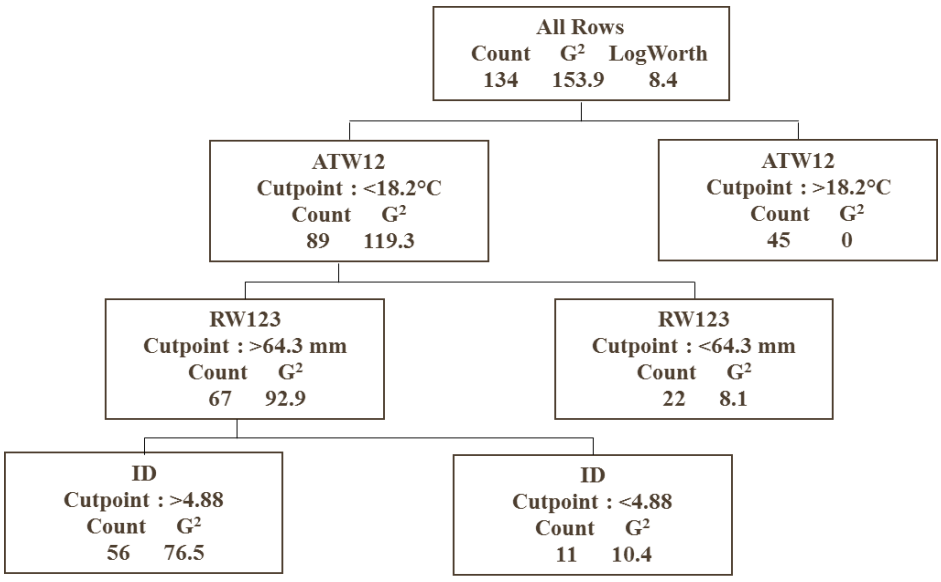

Figure 9. Decision tree for the prediction of a disease incidence ≥ 5%. The decision tree was constructed using a binary recursive partitioning analysis (JMP 13 Pro, SAS institute). The "disease" subgroup is represented in the left branches of the tree and the "healthy " subgroup is in the right branches. ATW12 is the average daily air temperature (°C) for the first two weeks after plantation. RW123 is the rain accumulation (mm) for the first three weeks after plantation. ID is the soil inoculum density within the experimental plot (ln ((spores/g dry soil) +1)). Count refers to the number of observations within each subgroup.

Table 7. Candidate G^2 and cut point value for each candidate (ID, ATW12 and RW123) at each split.

	ID[a]		ATW12[b]		RW123[c]	
Split	G^2 [d]	Cut point	G^2	Cut point	G^2	Cut point
First	19.4	3.86	34.6*	18.2	24.1	64.3
Second	10.8	3.86	3.5	13.9	18.3*	64.3
Third	6.0*	4.88	3	10.9	2.1	89.8

[a]Inoculum density, cut point is expressed in spores/g dry soil on a ln+1 scale

[b]Average daily air temperature for the first two week after plantation, cut point is expressed in °C

[c]Rain accumulation for the first three weeks after plantation, cut point is in millimeters of rain

[d]Candidate G^2

* Candidate that maximises the G^2 at each split

To assess the reliability of using ATW12 < 18.2°C, RW123 > 64.3 mm and ID > 4.88 (correspond to 132 spores/g dry soil) as predictive thresholds for a disease incidence ≥ 5%, a contingency table was constructed (Table 8). First, the results show that when assessed individually, each predictor gives good sensitivity (between 0.89 and 1.00) but poor specificity (between 0.42 and 0.45), while the overall accuracy is ranging between 0.56 and 0.60. Air temperature gives a best prediction (overall accuracy is of 0.60) than rain accumulation (overall accuracy of 0.57) or inoculum density (overall accuracy of 0.56) when assessed alone. When assessed two by two, the combination of predictors increases the overall accuracy (0.69-0.75) and the specificity (0.63-0.67) in all cases, while the sensitivity remains constant (0.89-0.97). Based on a combination of two predictors, the best prediction is made by the air temperature and rain accumulation, but only by an addition of 0.05 or 0.06 on the overall accuracy, when compared with the other combinations. Finally, the combination of the three predictors increases both the overall accuracy (0.79) and the specificity (0.76) while the sensitivity remains constant (0.89).

Table 8. Contingency table for assessment of using air temperature, rain accumulation and soil inoculum density as predictive parameters for Pythium stunt incidence.

Predictive Parameters[a]	Observed	Predicted P+	Predicted P-	Total	Overall accuracy[b]	Sensitivity[c]	Specificity[d]
ATW12	O+	35	0	35	0.60	1.00	0.45
	O-	54	45	99			
	Total	89	45	134			
RW123	O+	34	1	35	0.57	0.97	0.42
	O-	57	42	99			
	Total	91	43	134			
ID	O+	31	4	35	0.56	0.89	0.44
	O-	55	44	99			
	Total	86	48	134			
ATW12 + RW123	O+	34	1	35	0.75	0.97	0.67
	O-	33	66	99			
	Total	67	67	134			
ATW12 + ID	O+	31	4	35	0.70	0.89	0.64
	O-	36	63	99			
	Total	67	67	134			
RW123 + ID	O+	31	4	35	0.69	0.89	0.63
	O-	37	62	99			
	Total	68	66	134			
ATW12 + ID + RW123	O+	31	4	35	0.79	0.89	0.76
	O-	24	75	99			
	Total	55	79	134			

[a]ATW12 < 18.2°C, RW123 > 64.3 mm and ID > 4.88 (correspond to 132 spores/g dry soil) are considered predictive thresholds for having a disease incidence ≥ 5%

[b]Overall accuracy was calculated as the number of correct assessment (O+ & P+, and O- & P-) divided by the total number of assessments

[c]Sensitivity was calculated as the proportion of true positives (O+ & P+)

[d]Specificity was calculated as the proportion of true negatives (O- & P-)

3.5 Discussion

Pythium stunt caused by *P. tracheiphilum* leads, each year, to considerable yield losses for Quebec head lettuce growers. This problem is becoming more important as the majority of Canadian lettuce production is located in the Napierville county (Quebec, QC). However, despite the significance of the disease, the effect of soilborne inoculum density and weather parameters on Pythium stunt remains poorly documented. Hence, because no risk indicators are available to guide growers, they often resort to the use of chemicals (metalaxyl or cyazofamid) preventively, all over the growing season. Better knowledge related to factors (biotics and abiotics) that may influence the disease development is therefore required in order to rationalize pest control management of Pythium stunt.

Under controlled conditions, inoculum density of *P. tracheiphilum* was always well correlated with lettuce dry weight. As expected, cv. Prestige was more susceptible to infection by *P. tracheiphilum* than cv. Estival as the thresholds found for the previous is lower than that for the latter. Results from this study suggest that a concentration of 97 spores (cv. Estival) and, in average, 46 spores (cv. Prestige) per gram of dry soil was necessary to provide an infection resulting in a reduction of the total dry weight of lettuce by half. Similarly, Sauvage et al. (2007) compared severity of root rot caused by the oomycete *Aphanomyces euteiches* in pea plants grown in artificially infested soil with different inoculum density and found significant difference at concentration of 10 oospores/ml of soil when compared to non-inoculated control, which could be equivalent to 50 oospores/g of dry soil. Moreover, when assessing the effect of *P. tracheiphilum* soilborne inoculum density (nb sporangia/g soil) on two-week-old lettuces, Gracia et al. (1991) found that leaf and root weight of lettuces (cv. Ithaca) grown in artificially infested

soil with 200 spores/g soil were significantly lower when compared to non-inoculated controls. However, concentration below 200 sporangia/g soil were not tested.

In controlled-condition experiments, replicability of the experiment in time was possible for cv. Estival, but not for cv. Prestige since the time effect was significant for the second cultivar. A first explanation for the variability between both trials of cv. Prestige is related to the known sensitivity of the cultivar toward *P. tracheiphilum.* For a susceptible cultivar, small variations related to either strains virulence, spatial variation of inoculum density within pots or lettuce health during the previous two weeks of growth may have had more impact on the infection efficiency, if compared to a resistant cultivar, explaining the variation between experiment in time. However, each of these sources of variation remains hardly quantifiable and has not been investigated in this study.

At the highest soil inoculum densities (500, 1000 and 5000 sporangia/g dry soil), the increase in inoculum density (ID) had no more effect on lettuce weight in all trials, except for cv. Prestige in trial 1 where lettuce weight at 5000 sporangia/g dry soil is lower than at 1000 sp./g dry soil. Similar effect at high inoculum density has been observed for *Colletotrichum coccodes* causing black dot of potato, for which foliar symptoms, sclerotial density on roots or sclerotical development on stem remains constant above a threshold of soil inoculum density of 0.5-1.7 g of infected rye grain/l of soil (Nitzan et al. 2008).

Under field conditions, air temperature was negatively correlated and rain accumulation and soilborne inoculum density was positively correlated with DI in 2016 and 2017. For air temperature and rain accumulation, the first week after transplanting was always less correlated than the first two or three weeks, suggesting that favorable conditions need to be encountered during a period longer than 7 days. This might be

explained by a certain period required for mycelial root colonization by *P. tracheiphilum*. Jacquet (1979) found that *P. tracheiphilum* needed 9 days of incubation (at 18°C), to reach maximum mycelial growth, which was followed by a stationary phase. Therefore, favorable conditions encountered once this threshold of maximal growth of 9 days is reached would have a stronger influence on the disease development than favorable conditions encountered below this threshold, explaining why conditions occurring the first week were less correlated than those encountered thereafter. Beside explanations related to biological features, cultural practices could also be considered. In this study, lettuce transplants used by the different growers were grown in soil blocks under greenhouses until transplantation. Soil blocks were very compact and at the time of transplanting and only few roots had emerged from the block. Therefore, at the beginning of rooting, contacts of roots with the pathogen were less probable and probability would increase with subsequent root growth.

Since *P. tracheiphilum* is a soilborne pathogen, soil conditions were expected to be more influent than air conditions. However, soil temperature in this study were correlated with the disease in a lesser extent than for the air temperature (r = -0.58 for ATW12). The difference in the correlation coefficient for both parameter found in this study might be explained by the fact that, over the summer, less data for soil temperature were collected than for air temperature, and those data were also unevenly distributed over the temperature range in comparison to air temperature.

In 2016, both soil moisture and the rain accumulation showed significant correlation with disease incidence (STW12 is of 0.32 and RW12 is of -0.32), and thus evaluation of soil moisture would have been interesting to do in 2017 as well. However,

data and prediction for rain accumulation are readily available for lettuce growers unlike the percentage of volumetric water content of soil used to quantify the soil humidity, which justify the choice of rainfall instead of soil moisture for practical reasons. In 2016, soil pH was also collected in 62 experimental plots, but no correlation was found with disease incidence. Jacquet (1979) found that *P. tracheiphilum* growth on growing media was optimal between pH 5.5 to 6.5. Data for soil pH in this study were ranging between 5.09 and 6.73 with a mean of 5.92, which is within a close range of the pH value of 5.4 recommended for lettuce production by the Soil Chemistry and Fertility Commission (CRAAQ 2011). This, along with the small range of pH observed in this experiment, would explain the lack of correlation with disease incidence caused by *P. tracheiphilum.*

When assessed alone, air temperature (ATW12) below 18°C was found to be accurate to predict the disease 60% of the time, revealing that cooler temperature is optimal for the disease development. This is consistent with results obtain by Jacquet (1979), which found that growth, multiplication and sexual reproduction of *P. tracheiphilum* are optimal under 10 to 18°C, on growing media. Moreover, other pathogenic *Pythium* species are known to cause more severe disease at cooler temperature. For example, root infections of alfalfa caused by *Pythium. irregulare* were more severe between 16°C to 21°C, when compared with higher temperatures (Hancock 1991). Moreover, similar temperature optimals were observed for other oomycete species such as *Phytophtora ramorum* for which hyphal growth, sporulation and infection was shown to be optimal at temperature ranging between 15 and 20°C (Eyre and Garbelotto 2015).

Rain accumulation (RW123) above 64 mm was found to be accurate in predicting the disease incidence 57% of the time, revealing the importance of wet environment for the

disease development. Numerous studies reported the influence of soil moisture in the development of diseases caused by *Pythium* spp. (Biesbrock and Hendrix 1970; Bratoloveanu and Wallace 1985; Stanghellini and Burr 1973a). First, the rate of spore germination for *P. tracheiphilum* is known to be influenced by the time spent in water (Jacquet 1979). Moreover, *P. tracheiphilum* is one of the *Pythium* species capable of producing motile zoospores during favorable conditions (high moisture and presence of certain nutrient) (Jacquet 1979; Martin and Loper 1999). Those, in the presence of free water in the environment, are attracted by the roots exudates where they encyst and infect the plant (Martin and Loper 1999). Therefore, wet conditions may have stimulated the germination of spores as well as induced the production of zoospores and indeed increased the probability of infection as each sporangium may produce several zoospores (Jacquet 1979). A rain accumulation of 64 mm for three weeks corresponds to an average daily amount of rain of about 3.1 mm. For the region, such rain accumulation appears to be slightly higher than what is normally seen for the months of May and June since historical data shows a daily average of rain of 2.5mm in May, 2.6mm in June and 3.2mm in July (data covers 30 years, St-Bernard de Lacolle, QC, CAN, Latitude 45.1°, Longitude -73.4°(The Weather Network 2018)). As a comparison, in Florida, Campoverde et al. (2017) observed that an unusual daily average of rainfall of 0.157 inches (4 mm) for this region during the season 2015-16 resulted in an increase in disease incidence of 4.2% for Pythium-induced diseases and of 8.3% for Phytophtora-induced diseases, in ornamental plants. Similarly, it has been observed that Pythium stunt severity caused by *P. tracheiphilum* in lettuce fields of France increases in periods of high rainfall (Jacquet, 1979).

Soilborne inoculum density (ID) above 132 spores/g dry soil was found to accurately predict the disease 56% of the time. Gangneux et al. (2014) found a similar threshold for soil inoculum density under natural conditions for the oomycete *Aphanomyces euteiches* causing root rot in peas. They found a linear relationship between inoculum potential (IP) of the pathogen and the concentration of oospore in the soil, for which a concentration of 185 oospores/g dry soil corresponded to an IP of 3 (> 90% of the root system is brown without discoloration of epicotyl or hypocotyl).

When using air temperature and rain accumulation together, the accuracy of the prediction increases in such a way the overall accuracy reaches 75%. The addition of the soilborne inoculum density enhances the overall accuracy by 4% and the specificity by 9%. Accordingly, in a context where information concerning the soil inoculum would not be available, an accurate prediction of the disease incidence could be made based on the air temperature and the rain alone. However, soilborne inoculum density is a value that can be obtained prior to crop planting, unlike the weather prediction that is always susceptible to change. In this context, even if soilborne inoculum density does not exert a major influence on the prediction of disease, it provides a reliable tool in a decision-support system as it allows distinguishing between fields with higher risk from those with low risk of disease development before crop establishment.

In conclusion, the three hypotheses of the study were validated. A threshold of inoculum density under controlled conditions was defined for the widely used lettuce cultivar Estival and for the susceptible cultivar Prestige, however, with more variation in the response for the latter. Under field conditions, factors influencing the incidence of Pythium stunt were defined and can accurately be used to predict the disease incidence.

Moreover, different thresholds for soilborne inoculum density, air temperature and rain were set and proved to be good predictors of subsequent Pythium stunt disease in head lettuces grown in the Napierville county.

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3.7 Connecting text for chapter 4

In the previous chapter, relationship between soil inoculum density, weather parameters and Pythium stunt incidence have been evaluated in order to determine accurate predictors of disease incidence. However, biological activity within the soil is also known to modify the pathogenicity of soilborne pathogen. In the following chapter, the authors intended to characterize the microbial composition of soil rhizosphere of lettuces affected or not by Pythium stunt, and of lettuces grown in either a suppressive or conducive soil to *P. tracheiphilum*, in order to get a better understanding of the biological features that may influenced the development of Pythium stunt in head lettuce crops.

4. Characterization of soil suppressiveness to *Pythium tracheiphilum*

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4.1 Abstract

A next-generation, Illumina MiSeq based sequencing approach was used to characterize the bacterial, eukaryotic and fungal diversity of the rhizospheric soil of field-grown lettuce which presented symptoms of Pythium stunt versus healthy ones, and of lettuces transplanted in either a suppressive (SS) or conducive soil (CS) to *Pythium tracheiphilum*. This soilborne oomycete is responsible for Pythium stunt, a severe lettuce disease in the Napierville County (Québec, Canada). Bacterial and Eukaryotic compositions revealed significant difference between both soil classes. The suppressive soil was characterized by a higher number of potentially plant-associated taxa known for their pathogen-antagonistic effect or plant-growth promoting effect, notably the genus *Opitutus* (bacteria), the class of Phytomyxea (eukaryote) and *Leucosporidium drummii* (fungi) which were found in higher abundance in the suppressive soil. Moreover, more fungal species were specific to the suppressive soils in contrast with conducive soils. Results from this study suggest that the pathogenicity of *P. tracheiphilum* could be

modulated by the microbial composition surrounding the root system of lettuce plant, making possible the establishment of further integrated strategies that would promote the natural suppressive character of soils in the Napierville county.

4.2 Introduction

In Canada, lettuce is the most consumed fresh vegetable which generated more than 83 M$ in 2015 (ISQ and MAPAQ 2016). In this country, lettuces are primarily grown in the province of Quebec, mostly in the muck soil areas of the Napierville County where head lettuce crops are often affected by Pythium stunt, a disease caused by the soilborne oomycete *Pythium tracheiphilum*. The disease is responsible for important yield losses, which can reach up to 70% in certain cases (Van der Heyden, H. personal communication). Symptoms of Pythium stunt are characteristic: young plants are wilted and stunted because of water and nutrient deficiency due to infection of the root system. Moreover, a longitudinal cut down the taproot shows a yellow to reddish-brown discoloration of the tissues. Currently in Quebec, the control of Pythium stunt in head lettuce crops mainly rely upon the use of chemical fungicides (i.e. metalaxyl or cyazofamid). As no risk indicators are currently available to facilitate disease management, those chemicals are systematically applied preventively, all over the growing season.

Diseases management in sustainable agricultural systems should not be limited to the control of specific pest but should consider environmental as well as economic and social consequences of each remedial action (Chellemi et al. 2016; Katan et al. 2012). One of the four pillars proposed by Chellemi et al. (2016) for the development of a system-based approach aiming the management of soilborne diseases is to " incorporate activities into the cropping system designed to promote disease suppressive soil microbial

community ". According to its ability to suppress or not the pathogenic activity of a plant pathogen, soils may be classified as suppressive or conducive soils (Sullivan 2004). In fact, for two soils within which the pathogen is equally abundant, the one inducing the lowest disease incidence or severity is referred to as suppressive when compared to other soil. There are two known types of suppressiveness: general suppression, which is the general inhibition of a pathogen due to both biotic and abiotic factors (mainly based on competition for nutrient and space) and specific suppression, when the sole effect of one or selected groups of microorganisms such as antibiotic producer or parasite is responsible for suppressiveness (Weller et al. 2002).

Plenty of microorganisms, either bacterial or fungal, have been reported as being involved in the suppression of soilborne plant pathogen, including pathogenic species of *Pythium*. To name only a few, genus *Streptomyces* (El-Tarabily et al. 2010), *Rhizobium* (Bardin et al. 2004), *Pseudomonas* (Zhou and Paulitz 1993), *Burhkolderia* (Milus and Rothrock 1997) and *Bacillus* (Kipngeno et al. 2015; Peng et al. 2017; Zouari et al. 2016) are all bacteria involved in the suppression of Pythium-induced diseases. Moreover, some fungal organisms are known for their antagonistic activity toward *Pythium* spp., the most commons being *Trichoderma* sp. (Chet et al. 1981; Harman et al. 2004; Howell 2002), *Gliocladium* sp. (Chet 1981), *Penicillium* sp. (Nicoletti and De Stefano 2012) and the non-pathogenic *Pythium oligandrum* and *P. nunn* (AL-Hamdani et al. 1983; Paulitz and Baker 1988).

The use of metagenomic analysis, such as next-generation sequencing (NGS), to understand complex soil ecosystems is increasing in the field of plant pathology, as more and more organisms are being sequenced (Wakeham and Pettitt 2017). This powerful tool

can notably be used to investigate the role of organisms in disease suppression and allows getting a more holistic view of the organisms involved in disease suppression. For example, metagenomic approach has recently been used to analyse soil suppressive to *Rhizoctonia solani* and highlighted the implication of Proteobacteria, Firmicute, Actinobacteria and Bacterioidetes in *R. solani* suppressiveness (Chapelle et al. 2016; Mendes et al. 2011).

In the light of these insights, the assessment of soil suppressiveness toward *Pythium tracheiphilum* by identifying naturally occurring microorganisms may provide knowledge to integrate in the development of a system-based approach to *P. tracheiphillum* management. Therefore, the aim of this study was to investigate the microbial diversity of soil from the root zone of lettuce plants, using metagenomic analysis. More specifically, the objectives were to compare the bacterial, eukaryotic and fungal composition (overall richness or/and relative abundance of certain taxa) of rhizospheric soil of i) lettuces showing symptoms of Pythium stunt compared to healthy ones and ii) lettuces grown in a suppressive soil (SS) versus a conducive soil (CS) to *P. tracheiphilum*.

4.3 Materials and methods

4.3.1 Data collection

4.3.1.1 Sampling

In 2016, six 25 m^2 experimental plots, each containing 192 head lettuce plants, were installed in three different fields (45°07'28.1"N 73°32'17.7"N; 45°14'53.6"N 73°42'12.6"N; 45°14'41.6"N 73°42'03.3"N) located on two farms in the Napierville county (Qc, Canada). In order to quantify *P. tracheiphilum* inoculum density within each experimental plot, bulk soil samples were taken prior to planting, placed in plastic bags and

frozen (-20°C) until use. Each soil sample was made from 15 random soil subsamples taken with an auger in the first 15 centimeters from the soil surface. Within each experimental plot, lettuce plants were also collected. Whole lettuce plants were uprooted and gently shaken to remove non-adhered soil from the roots. Then, soil adhering to the roots (rhizospheric soil) was collected by shaking roots through a sieve. Two different rhizospheric soil samples were collected for each experimental plot; the first was made of nine healthy plants taken in three different parts within the plot and the second was made of nine diseased plants (showing symptoms of Pythium stunt) taken in three different parts within the plot. In summary, within each of the six experimental plots, one bulk soil sample, one rhizospheric soil sample from healthy plants and one rhizospheric soil sample from infected plants were collected. Sampling and planting dates are given in table 9.

4.3.1.2 Evaluation of disease incidence and weather data collection

Disease incidence was evaluated twice during the growing season. At each evaluation the number of symptomatic plants (wilted or stunted plants and for which the root xylem showed yellow-brown discoloration after a longitudinal cut down) within each plot was counted. Diseased plants were removed from the plot during the first evaluation to avoid recounting them during the next evaluation. Sums of all evaluations were used to calculate the percentage of disease incidence (DI) (DI= (number of diseased plants / total number of plants) *100). The total number of plants refers to the total number of lettuces planted within the experimental plot before any evaluation.

Air temperature (°C) and rainfall (mm) were collected hourly from weather stations (Watchdog 2700, Spectrum Technologies Inc., Illinois, USA) located outside the field within a 5 kilometers radius.

4.3.2 DNA extraction and PCR analysis

DNA extraction from bulk and rhizospheric soil samples was made on 0.20 g of dry soil using the FastDNA SPIN kit for soil (MP Biomedicals, USA), following instructions from the manufacturer. Quantity and purity of the collected DNA was measured by spectrophotometry using a Nanodrop lite instrument (Thermo Fisher Scientific, Mississauga, Ontario, CAN).

Quantification of *Pythium tracheiphilum* soilborne inoculum density was performed using a real-time Taqman PCR protocol described by Van der Heyden et al. (In press). Details on the procedure have previously been described in section 3.3.2.2.

4.3.3 Libraries, sequencing and bioinformatic

Metagenomic analysis was conducted on the rhizospheric soil samples to evaluate the diversity for Bacteria, Eukaryote and Fungi. First, validation of inhibition and a dilution test were performed prior to the libraries preparation. These tests were done to detect the possible presence of PCR inhibitors that sometime remain after DNA extraction. When PCR inhibition is detected, then the dilution of the sample is required to minimize the effect of the PCR inhibitor. The amplification of the V6-V8 regions from the 16SrRNA (bacteria), 18SrRNA (eukaryota) and ITS1 region (fungi) was performed using universal primers, as described by Comeau et al. (2011) and McGuire et al. (2013), and using a two-step dual-indexed PCR approach specifically designed for Illumina MiSeq instruments by the Plateforme d'analyse génomiques (Institut de Biologie Intégrative et des Systèmes (IBIS), Laval University, Québec, QC, CA). Amplicons were paired-end sequenced with 300 bases (2X 300bp) reading. Notice that even if fungus was also amplified by the 18SrRNA region

used to amplify the total eukaryota, all the analysis related to the fungus was based on the results given by the ITS amplification.

4.3.4 Bioinformatic data processing

Bioinformatic data processing was performed by the Laboratoire d'Écologie Microbienne (LEM) at the Institut de Recherche et de Développement en Agroenvironnement (IRDA, Québec City, QC, CAN.) and involved different strategies for data processing including steps such as validation quality, baseline, microbial richness index and microbial diversity measurements. The SILVA (version 119) (Quast et al. 2013) baseline was used for bacterial and eukariotic analyses of diversity, and the UNITE (Kõljalg et al. 2013) baseline was used for the evaluation of the fungal species diversity. Filtering of the Operational Taxonomic Units (OTUs) obtained was performed prior to the diversity analysis, in order to reduce the possible issues related to OTUs found in low abundance.

4.3.5 Comparison between diseased and healthy plants

In this first part, using data from the metagenomic analysis, microbial community comparison was made between affected plants samples (n=6) and healthy plants samples (n=6), in order to determine if the infection level of the plants would be due to differences in the microbial composition of the soil from their root zone. Principal Coordinates Analysis (PCoA) (R, R core team project 2014) was conducted to compare the microbial composition. In the PCoA analysis, a distance matrix based on the Bray-Curtis index, which is widely used in microbial ecology, was used to evaluate the distance (pairwise) between each sample. 2D graphical representation of the two principal components of the

PCoA analysis was also constructed. A Permanova analysis (R, R core team project 2014) was also performed on the Bray-Curtis distance matrix. This non-parametric multivariate analysis of variance was used to assess the significant difference in the microbial composition between each class (affected vs healthy plants).

4.3.6 Comparison between suppressive and conducive soil

In this second part, the 12 subplots were divided into two classes, based on *P. tracheiphilum* soilborne inoculum density and on the percentage of disease incidence (Table 10). As shown in table 10, following a student *t*-test analysis, there was no significant differences between the two classes for inoculum density and environmental conditions. However, disease incidence was significantly higher (2.5 times higher) in one class compared to the other. Therefore, according to the definition of a suppressive soil, the class presenting lower disease incidence was defined as suppressive soil (SS) and the class presenting higher disease incidence was defined as conducive soil (CS). Then, comparisons of the microbial communities for bacteria, eukaryota and fungi were made between SS and CS by conducting a PCoA analysis and Permanova analysis, as described earlier.

Two different microbial diversity indexes, the Chao1 index and the Shannon index, were used to compare the diversity between SS and CS class and were estimated from a common number of 5000 sequences for the bacteria and eukaryote, and 9000 sequences for the fungi. The Chao1 index was used to estimate the number of non-observed OTUs, from the number of observed OTUs, to get an estimation of the alpha diversity (number of observed *species* within the sample). The Chao 1 index was calculated as follow:

$$S_1 = S_{obs} + \frac{F_1^2}{2F_2}$$

where S_1 is the Chao1 index, S_{obs} is the number of OTUs in the sample, F_1 is the number of OTUs with only a single occurrence in the sample (singletons) and F_2 is the number of OTUs with exactly two occurrences in the sample (doubletons).

Second, the Shannon index, which involves both the number of different OTUs and their relative abundance, was used to evaluate the diversity according to the evenness of the OTUs distribution. For example, a maximal diversity value would be reached if all the OTUs found in a sample have the same relative abundance. The Shannon index was calculated as follow:

$$H' = -\sum pi \, ln \, p_i$$

where *H'* is the Shannon index, and p_i is the proportion of clones in the *i*th OTU.

For fungi, bacteria and eukaryote, the relative abundance of taxa at different taxonomic level was made for each sample. The relative abundance was calculated as the proportion of OTU(s) belonging to the taxa compared to the total number of OTUs within the sample. A Welch *t*-test (SAS Proc GLM, SAS institute) was performed, at each taxonomic level, on taxa with a relative abundance higher than 1% and on taxa being at least 2 times more abundant in a class compared to the other. Every other taxa were not tested for mean comparisons.

Table 9. Sampling and plantation date.

Farm	Field	Plot	Bulk soil sampling date	Planting date	Lettuce sampling date[a]
1	31	A	May 12th	May 27th	June 24th
		B	May 12th	May 27th	June 24th
	21	A	May 12th	May 21th	June 24th
		B	May 12th	May 21th	June 24th
2	35	A	June 3th	June 3th	July 5th
		B	June 3th	June 3th	July 5th

[a]Refer to the rhizospheric soil samples

Table 10. Classification of the experimental plots as suppressive or conducive soil.

	Suppressive soil class	Conducive soil class	t-test[e]
Disease incidence [a]	11%	27%	**<0.0001**
Inoculum density [b]	264.7	218.7	0.5005
ATW12[c]	16.7	16.7	0.9153
RW123[d]	53.9	40.5	0.4023

[a] Percentage of symptomatic plant within the plot, mean of n = 6 in each class

[b] Number of oospores/g dry soil, mean of n = 6 in each class

[c] Daily average of air temperature for the first two weeks after plantation, mean of n = 6 in each class

[d] Rain accumulation (mm) for the first three weeks after plantation, mean of n = 6 in each class

[e] Pairwise comparison between suppressive and conducive soil classes

4.4 Results

4.4.1 Comparison of the microbial composition

4.4.1.1 Comparison between diseased and healthy plant

In this section, comparisons were made between the microbial composition of the rhizospheric soil of diseased plants (composite sample of 9 lettuces) affected with Pythium stunt (n=6) and healthy plants (n=6). As shown in figure 10, no distinction between microbial composition can be made for bacteria, nor for eukaryotes and fungi as the result of the Permanova analysis, which indicated no significant differences for the microbial diversity between both classes.

4.4.1.2 Comparison between suppressive and conducive soils

In this second part, comparisons between SS samples and CS samples were made. For the bacterial community, the PC1 axis from the PCoA plot explained 30.5% of the total variation and PC2 2 axis explained 18.8% of the total variation between samples. The result of the Permanova analysis suggested a significant difference between the suppressive soil and the conducive soil ($P = 0.037$) (Figure 11a).

For the eukaryotic community, the PC1 axis from the PCoA plot explained 27.2% of the total variation and PCoA 2 axis explained 18.2% of the total variation between samples. The result of the Permanova analysis also indicates a significant difference between the suppressive and conducive soil ($P = 0.011$) (Figure 11b).

For the fungal community, the PC1 axis from the PCoA plot explained 42.6% of the total variation and PC2 axis explained 21.5% of the total variation between samples.

However, the result of the Permanova analysis suggested that there was no significant difference between the suppressive and the conducive soil at the 0.05 level ($P = 0.077$) (Figure 11c).

Since significant difference was only found when the SS and CS classes were compared, microbial diversity analysis as well as comparison of the relative abundance of taxa was conducted for SS and CS classes only, and not for " diseased " and " healthy " plants samples.

4.4.2 Microbial diversity

When the Chao1 index was used, similar microbial diversity was found between SS and CS, for bacteria, eukaryote and fungi (Figure 12a, b and c). When the Shannon index was used, significant differences could be found for fungi ($p = 0.001$) where higher Shannon index value was found in the SS when compared to the CS class (Figure 13c). However, no difference was observed for bacteria ($p = 0.867$) neither for eukaryotes ($p = 0.889$) (Figure 13a and b, respectively).

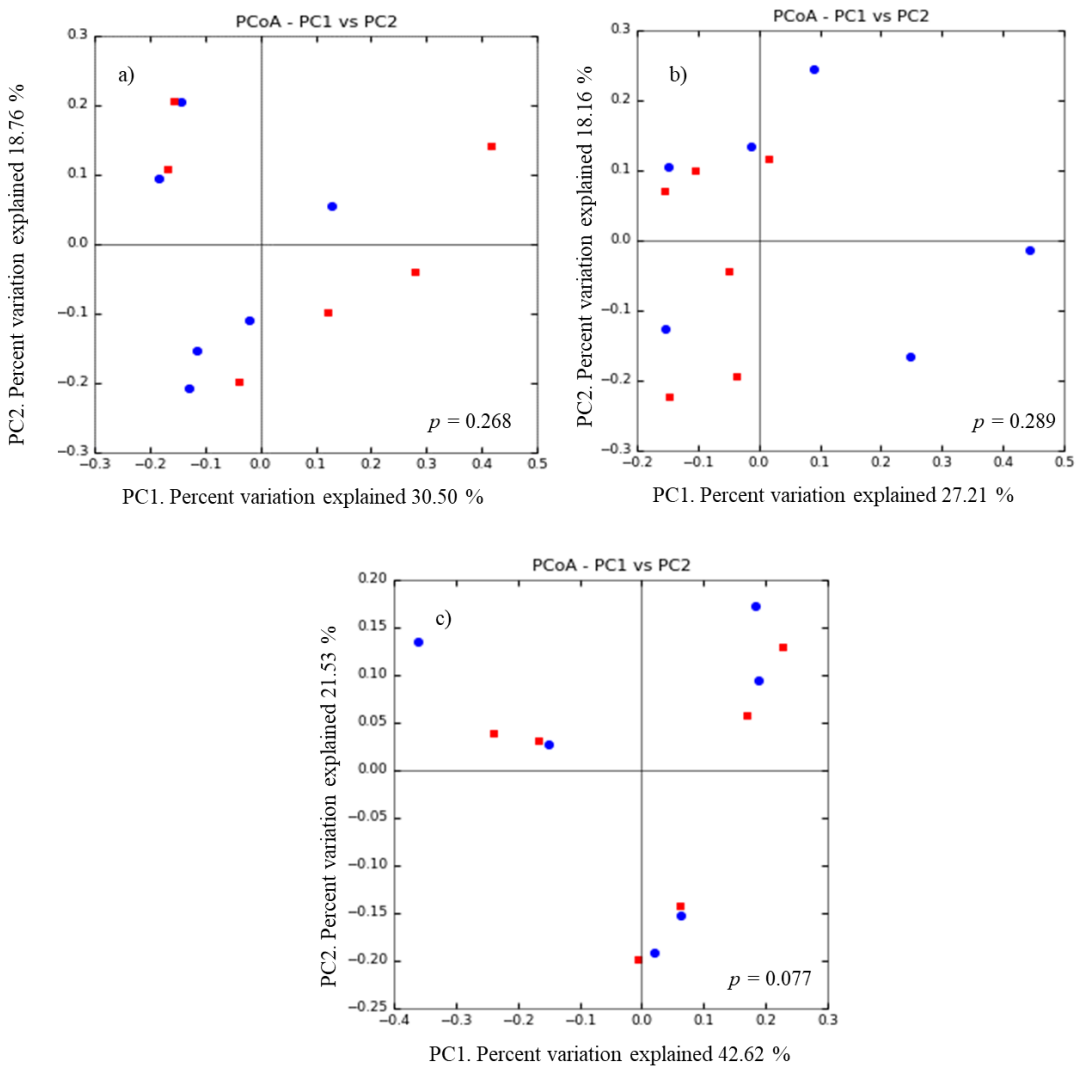

Figure 10. 2D representation of the PCoA analysis comparing healthy and diseased lettuces for a) bacteria b) eukaryotes and c) fungi. Red points are diseased lettuces and blue points are healthy lettuces.

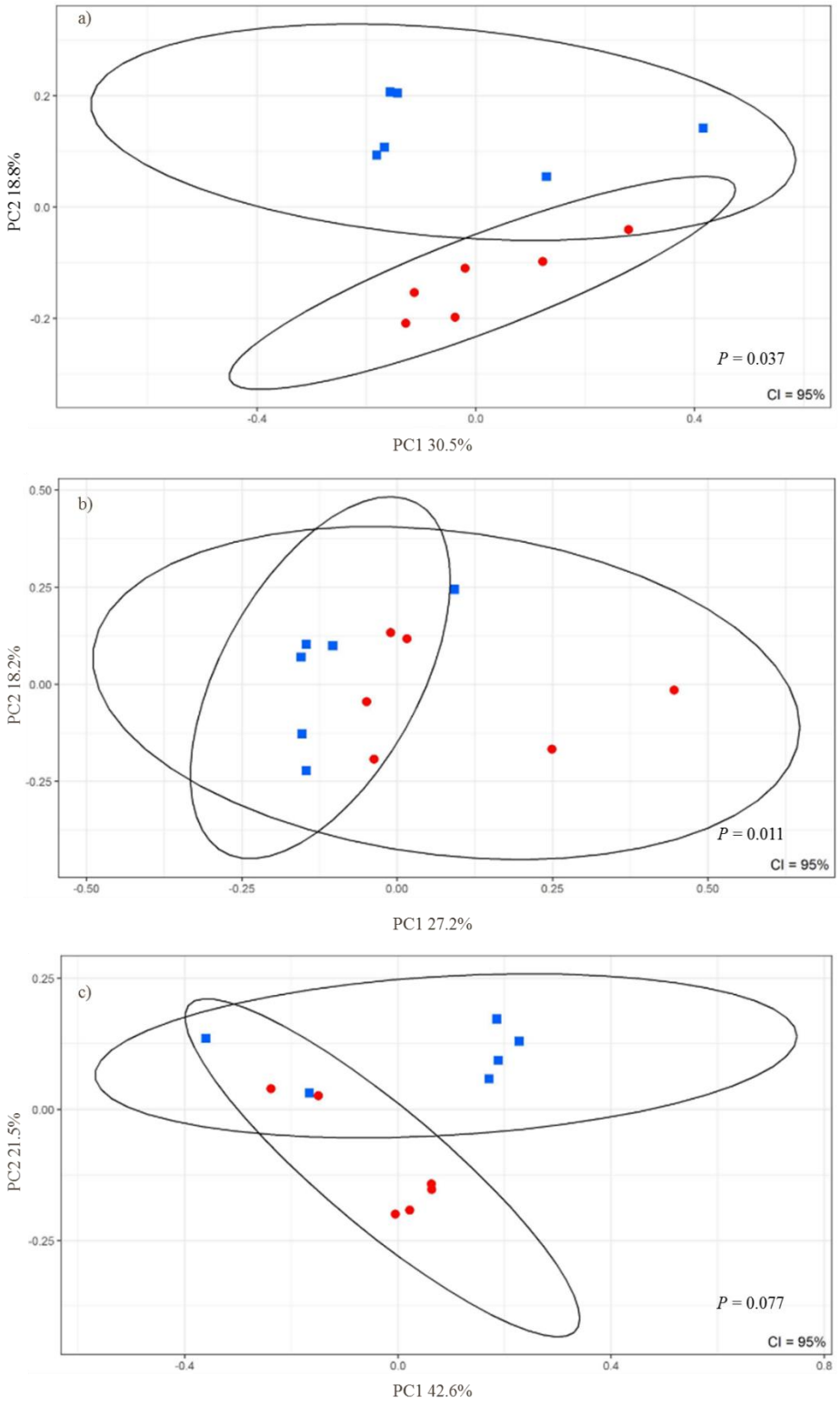

Figure 11. 2D representation of the PCoA analysis comparing suppressive and conducive soils for a) bacteria b) eukaryotes and c) fungi. Red points are conducive soil samples and blue points are suppressive soil samples.

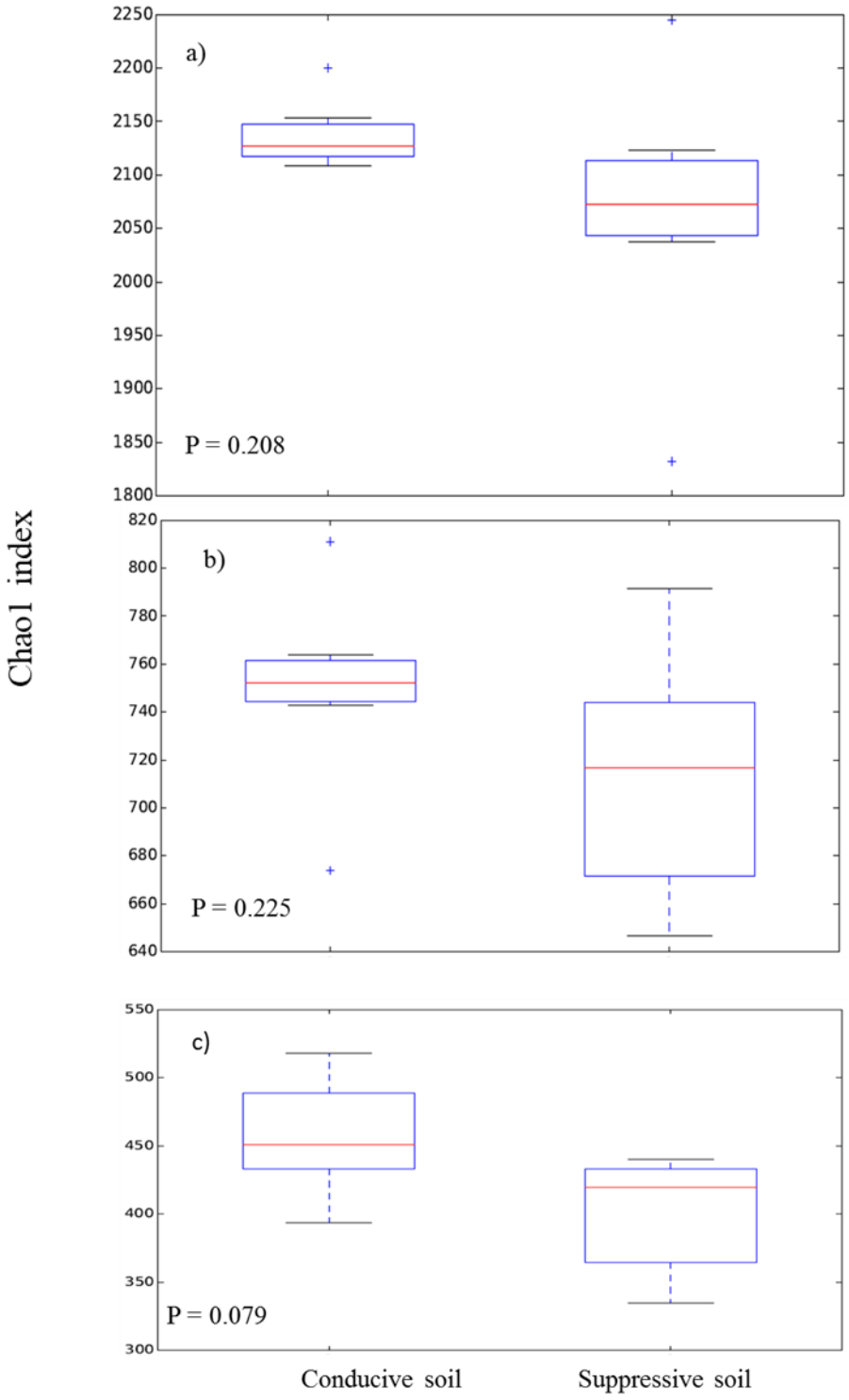

Figure 12. Microbial diversity given by the Chao1 index for a) bacteria, b) eukaryote and c) fungi, for conducive and suppressive soil.

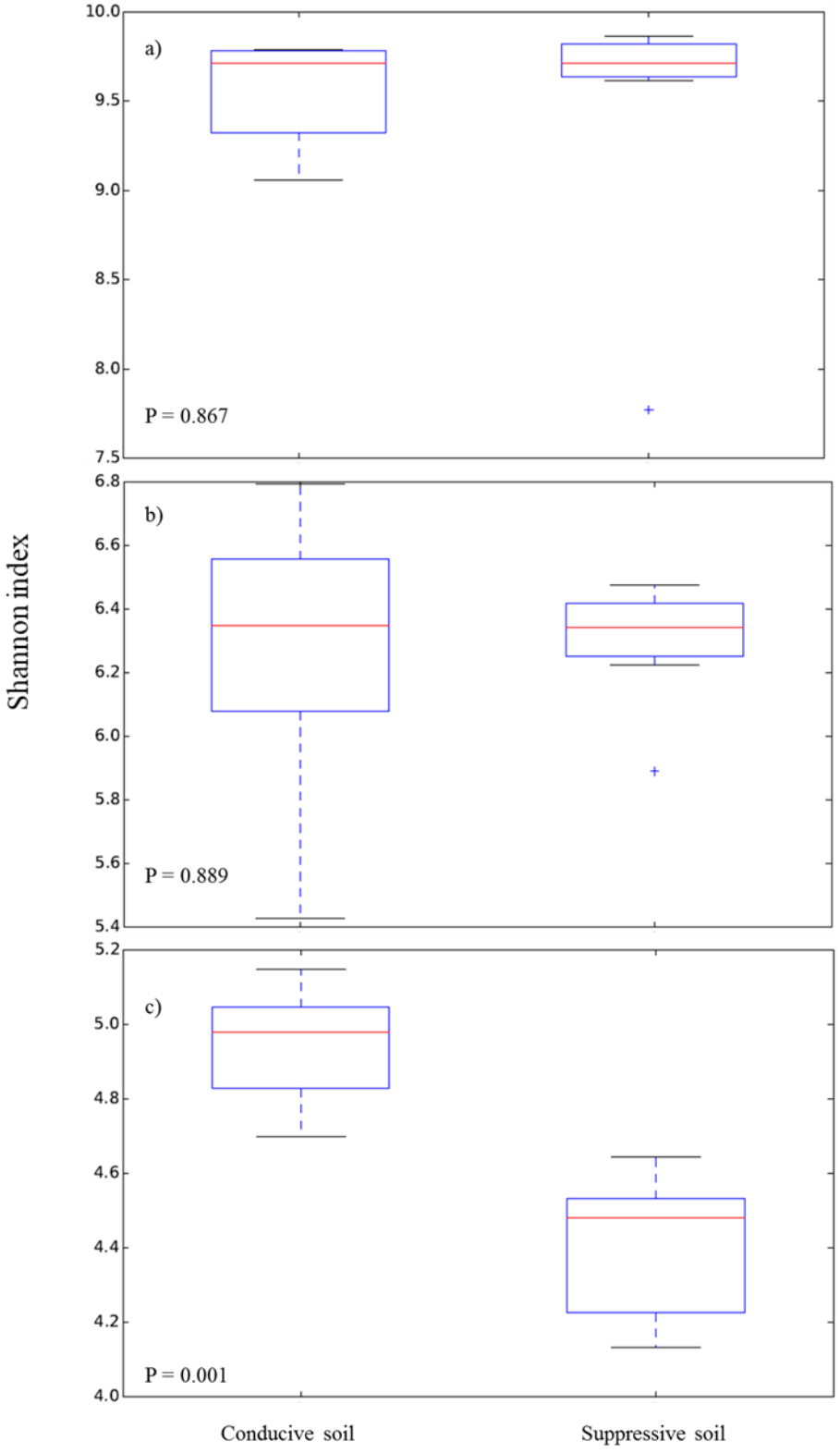

Figure 13. Microbial diversity given by the Shannon index for a) bacteria b) eukaryote and c) fungi for suppressive and conducive soil.

4.4.3 Relative proportion of taxa

For bacteria, at the first taxonomic level, Actinobacteria, Proteobacteria and Chloroflexi were the three most abundant taxa for both the SS and the CS class, each accounting for 10% or more of the total abundance and are seen in the same order of predominance (Table 11). However, the other phyla followed a different order of predominance in the SS than in the CS class. Firmicutes were in fourth place in the SS class (relative abundance of 7.82%) while they were in the sixth place in CS (relative abundance of 3.42%). For both classes, five taxa were observed in lower than 10% but remains higher than 1%, and 16 taxa were recovered in low abundance (< 1%) for which 11 of them were lower than 0,01% (Table 11). At a deeper taxonomic level, the Micrococcales order was the most abundant within the Actinobacteria with a predominance for the genus *Arthrobacter* while the Rhizobiales order was the most abundant within the Proteobacteria (alpha-Proteobacteria). The KD4-96 class was the most abundant within the Chloroflexi and Bacillales order was the most abundant within Firmicutes with a prevalence for the genus *Bacillus* (Figure 14).

Results from the Welch's t-test analysis revealed significant differences in the relative abundance between SS and CS classes for the Chloroflexi phylum ($P = 0.011$, 10.37% for SS and 14.55% for CS class) and for the Gemmatimonadales order ($P = 0.017$, 3.46% for SS and 4.82% for CS class). Significant differences were also observed for certain groups with a relative abundance lower than 0.1%. The order of Methylophilales was 4.2 time more abundant in the SS than in CS ($P = 0.034$), the Rhodobacterales order was 2.4 time less abundant in the SS than in the CS ($P = 0.006$) and the *Opitutus* genus

(Verrucomicrobiota) was 2.2 time more abundant in the SS than in the CS class ($P = 0.042$) (Table 12).

Certain bacterial genera were recovered in at least three SS samples, in low abundance (all were < 1 %), but were not recovered in any of the CS samples. Those genera are *Amnibacterium*¸ *Mucilaginibacter* and *Solimonas.* In the same way, uncultured species from the Sandaracinaceae family were recovered only in the CS class (Table 12).

For eukaryotes, at the first taxonomic level, Nucletmycea was the most abundant taxa followed by Holozoa, Rhizaria, Alveolata and Stramenopiles, in both classes (Table 13). At a deeper taxonomic level, the fungi were predominant within the Nucletmycea, the Animalia (kingdom) was the predominant phyla within the Holozoa, with a prevalence of the Nematoda, Annelida and Arthropoda phyla, the Cercozoa phylum was predominant within the Rhizaria, with a prevalence for the genus *Heteromita*, the Ciliophora phylum was predominant within the Alveolata, with a prevalence for the subclass Hypotrichia and the Ochrophyta phylum was predominant within the Stramenopiles with a prevalence of the genus *Spumella* (Figure 15).

Results from the Welch's t-test analysis revealed significant differences in the relative abundance between SS and CS classes for some groups of eukaryotes. The relative abundance of Phytomyxea was higher in SS than in the CS class and the Tribonematales order as well as the Bacillariophytina order was higher in the CS than in the SS class (Table 14)

Some eukaryotic genera or species were recovered in at least three SS samples in low abundance (<1 %) but were not recovered in any of the CS samples. Those are the

plant pathogenic species *Phytophtora infestans* (Pythiaceae family), and uncultured genera from the Chrysophycea family. In the same way, some genera or species were recovered in at least three CS samples, but not in any of the SS samples. Those are the nematode *Fridericia tuberosa* and *Thalassiosira* sp. (Table 14).

For the fungi, at the first taxonomic level, Ascomycota was the most abundant taxa, followed by Basidiomycota and Chytridiomycota. Each of these taxa followed the same order of predominance in both classes (Table 15). Several fungi were classified as unidentified fungi and resulted in 11.5% (SS) and 13.5% (CS) of the total fungal abundance. At a deeper taxonomic level, *Gibellulopsis chrysanthemi* (14.0% in SS and 10.86% in CS), *Fusarium acutatum* (11.54% in SS and 12.42% in CS), *Cephalosporium* sp. (5.60% in SS and 6.34% in CS), *Penicillium levitum* (3.65% in SS and 1.72% in CS) and *Penicillium brasilianum* (1.60 % in SS and 0.04% in CS) were the most abundant species (represent >1%) within the Ascomycota. The total abundance of Basidiomycota was significantly higher in the suppressive than in the conducive soil ($P = 0.0039$). Within this phylum, Tremellomycetes was predominant where the species *Cryptococcus laurentii* was the most abundant. This species was found to be 10-fold more abundant in the suppressive than in the conducive soil, though not significantly (2.26% in SS and 0.22% in CS). Within the Zygomycota, *Mortierella* sp. (1.47% in SS and 1.14% in CS) was the most abundant genus (Figure 16). However, none of the latter species was significantly in higher abundance in one class compared to the other.

Results from the Welch's t-test analysis revealed that some genera or species were significantly more abundant (at least 2 times higher) in the CS class than in the SS class but represented less than 1% of the total fungal abundance. Those were *Ulocladium*

chartarum, Arachnopeziza obtusipila, Cryptoccoccus terreus and *Guehomyces pullulans.* In the SS class, only *Leucosporidium drummii* were more abundant than in the CS class (Table 16).

A total of 17 different species (unidentified genera excluded) were recovered in at least three of the six SS samples but were not recovered in any CS samples. The genus *Rhizoscyphus* sp. was recovered in five of the six SS samples, *Byssochlamys zollernial, Leotyomycete* sp., *Blastobotrys mokoenaii* and *Trichosporon dehoogii* were recovered in four of the six SS samples, and *Stagonosporopsis crystalliniformis, Byssochlamys spectabilis, Talaromyces* sp., *Oidiodendron rhodogenum, Candida subhashii, Nadsonia starkeyo-henricii, Pochonia bulbilosa, Mycothermus thermophilu, Vankya heuflen, Rhizophlyctis rosea, Rozellomycota* sp. and *Umbelopsis* sp. were recovered in three SS of the six samples (Table 17).

In the same way, a total of 10 different species (unidentified genera excluded) were recovered in at least three of the six CS samples but were not recovered in any SS samples. The species *Remersonia thermophila* was recovered in five of the six CS samples, *Ustilago maydis, Aspergillus pseudodeflectus, Penicillium chrysogenum* and *Chrysosporium* sp., were recovered in four of the six samples, and *Chaetomium carinthiacum, Ochroconis tshawytschae, Bipolaris eleusines, Microascus brevicaulis* and *Aspergillus austraoafricanus* were recovered in three of the six CS samples (Table 17).

Table 11. Bacterial phylum (first taxonomic level) ranked in order of their predominance within suppressive and conducive soil classes.

	Suppressive soil			Conducive soil		
Predominance	**Taxa**	**%[a]**	**σ [b]**	**Taxa**	**%**	**σ**
1	Actinobacteria	41.59	3.69	Actinobacteria	40.89	5.17
2	Proteobacteria	22.31	5.37	Proteobacteria	19.97	2.16
3	*Chloroflexi	10.37	1.62	*Chloroflexi	14.55	2.38
4	Firmicutes	7.82	13.66	Acidobacteria	8.91	2.01
5	Acidobacteria	7.03	1.60	*Gemmatimonadetes	4.89	0.87
6	*Gemmatimonadetes	3.52	0.61	Firmicutes	3.42	4.67
7	Bacteroidetes	3.12	1.35	Bacteroidetes	2.72	0.72
8	Verrucomicrobia	1.69	0.53	Verrucomicrobia	1.60	0.30
9	Other	0.80	0.20	Nitrospirae	0.95	0.26
10	Nitrospirae	0.61	0.14	Other	0.92	0.15
11	Planctomycetes	0.58	0.17	Planctomycetes	0.51	0.07
12	Candidate division WS3	0.11	0.06	JL-ETNP-Z39	0.16	0.03
13	Cyanobacteria	0.10	0.05	Candidate division WS3	0.14	0.04
	Other ≤ 0.1%[c]	0.35	0.23	Other ≤ 0.1%	0.39	0.20
Total		100.00			100.00	

[a] Percentage of relative abundance (mean of 6 samples)

[b] Standard deviation of the mean

[c] Represent 11 taxa

*Indicate significant difference

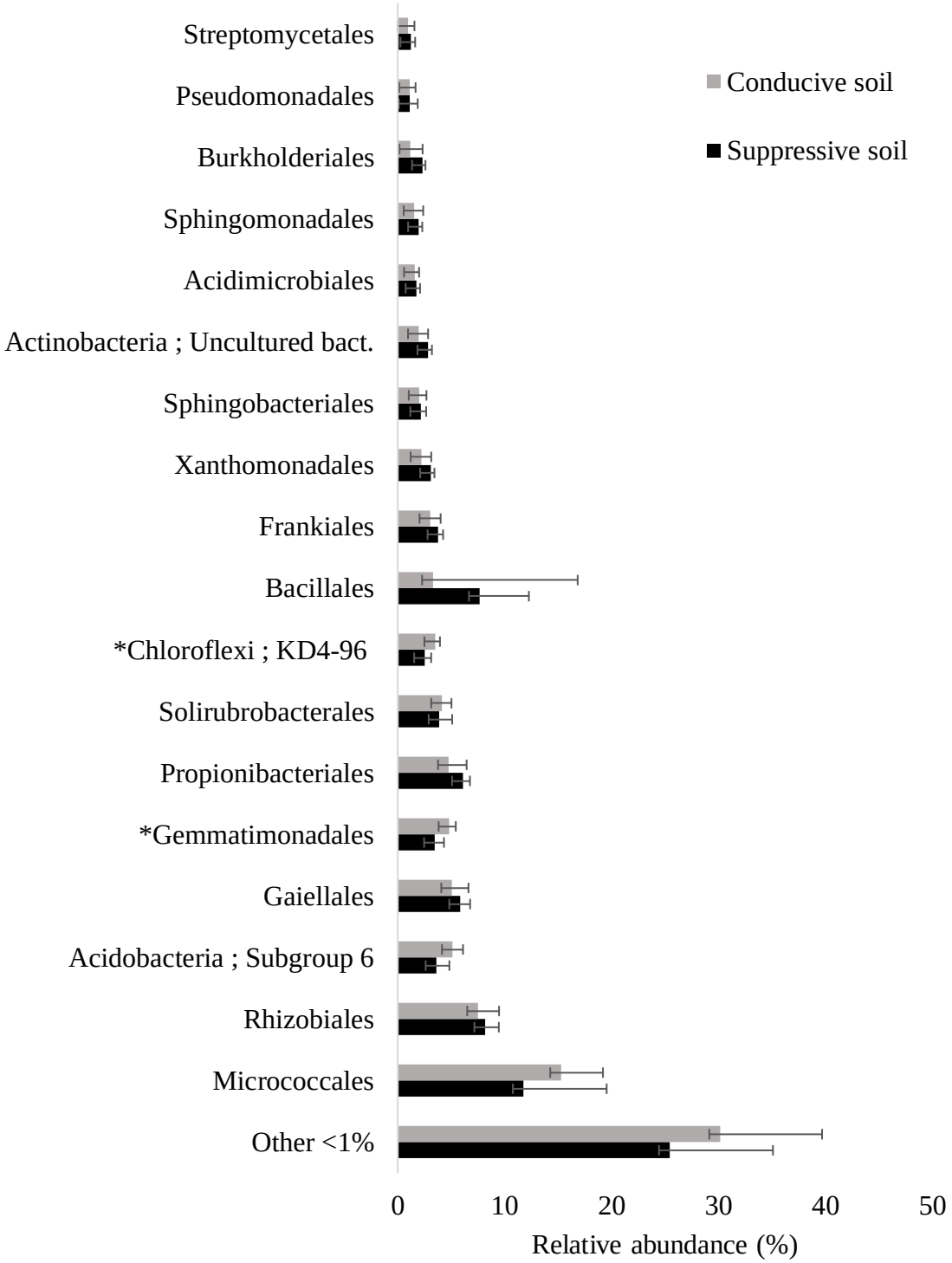

Figure 14. Relative abundance of the most abundant bacterial order (>1%) found in suppressive and conducive soil. * The relative abundance is significantly different between suppressive and conducive soil, based on a Welch's t-test analysis. Bars are standard deviation of the mean (n=6).

Table 12. Significant difference in the relative abundance of bacterial genera (%) ± standard error of the mean between suppressive (n=6) and conducive soils (n=6) to *Pythium tracheiphilum*, and possible functions of species belonging to the same genus reported in the literature.

			Soil classes				
Phylum	**Family**	**Genus**	**Suppressive**	**Conducive**	**Increase in suppressive soil**	**p value**	**Possible functions (reference)**
Actinobacteria	Microbacteraceae	*Amnibacterium*	0.012± 0.014	0	.	.	/
Bacteroidetes	Sphingobacteracea	*Mucilaginibacter*	0.029± 0.029	0	.	.	Plant growth promoter (Madhaiyan et al. 2010)
Deltaproteobacteria	Sandaracineae	uncultured	0	0.011± 0.012			/
Gammaproteobacteria	Solimonadaceae	*Solimonas*	0.01± 0.011	0	.	.	/
Verrucomicrobia	Opitutaceae	*Opitutus*	0.457± 0.212	0.208± 0.054	2.2x	0.042	Plant-beneficial bacteria, biocontrol (Li et al. 2015)

Table 13. Eukaryotic division (second taxonomic level) ranked in order of their predominance within conducive and suppressive soil classes.

	Suppressive soil			Conducive soil		
Predominance	**Taxa**	**%[a]**	**σ[b]**	**Taxa**	**%**	**σ**
1	Nucletmycea	60.54	13.05	Nucletmycea	50.16	9.84
2	Holozoa	19.86	13.05	Holozoa	32.82	13.49
3	Rhizaria	13.58	2.59	Rhizaria	12.68	2.89
4	Alveolata	5.02	1.74	Alveolata	3.15	1.29
5	Stramenopiles	0.97	0.17	Stramenopiles	1.14	0.31
6	Freshwater Opisthokonta	0.03	0.04	Freshwater Opisthokonta	0.05	0.02
Total		100.00			100.00	

[a]Relative abundance (Mean of 6 samples)

[b]Standard deviation of the mean

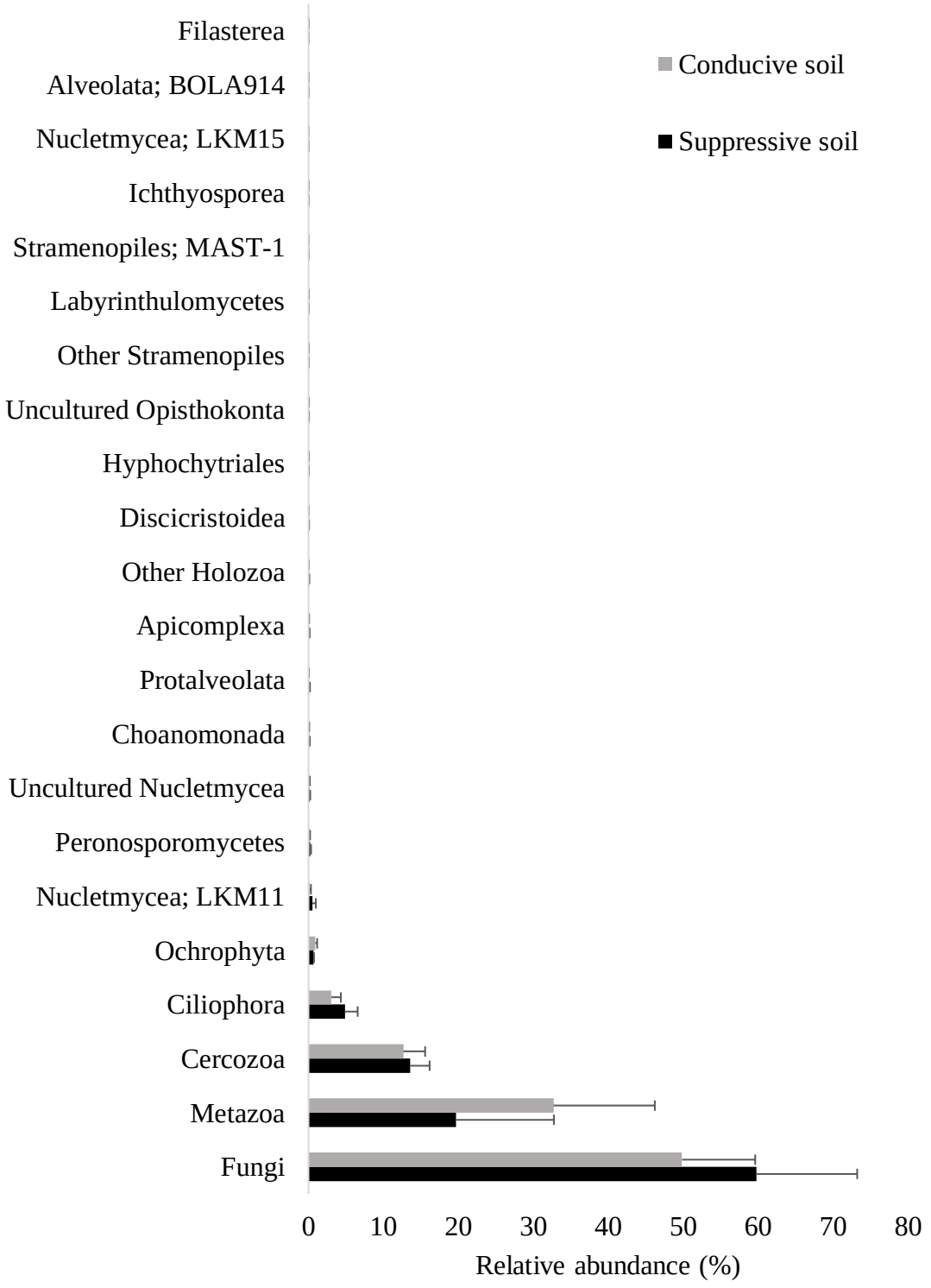

Figure 15. Relative abundance of the eukaryotic phylum found in suppressive and conducive soil. Bars are standard deviation of the mean (n=6).

Table 14. Significant differences in the relative abundance of eukaryotic genera (%) ± standard error of the mean (n=6) between suppressive and conducive soils to *Pythium tracheiphilum*, and possible functions of species belonging to the same genus reported in the literature.

				Soil class				
Class	**Order**	**Genus**	**species**	**Suppressive**	**Conducive**	**Increase or decrease**	***p*-value**	**Possible functions (reference)**
Enchytridae	Annelida	*Fridericia*	*tuberosa*	0	0.16± 0.136	.	.	/
Phytomyxea	.	.	.	0.571± 0.243	0.261± 0.098	2.2x	0.035	Parasite of Pythium (Dylewski and Miller 1984)
Diatomea	Bacillariophytina	.	.	0.024± 0.023	0.131± 0.059	5.4x	0.008	/
		Thalassiosira	*sp.*	0	0.023± 0.032	.	.	/
Oomycete	Peronosporales	*Phytophtora*	*infestans*	0.02± 0.022	0	.	.	Plant pathogen
Xanthophycea	Tribonematales	.	.	0.034± 0.017	0.095± 0.049	2.8x	0.039	/

Underlined when the relative abundance is lower in the suppressive soil

Table 15. Fungal division (first taxonomic level) ranked in order of their predominance within conducive and suppressive soil classes.

	Suppressive soil			Conducive soil		
Predominance	**Taxa**	**%[a]**	**σ[b]**	**Taxa**	**%**	**σ**
1	Ascomycota	78.61	5.09	Ascomycota	78.03	4.78
2	Unidentified	11.50	4.93	Unidentified	13.51	5.39
3	*Basidiomycota	6.67	0.85	*Basidiomycota	4.86	0.63
4	Zygomycota	2.77	0.49	Zygomycota	3.08	1.00
5	Chytridiomycota	0.44	0.33	Chytridiomycota	0.52	0.34
6	Rozellomycota	0.00	0.01	Cercozoa	0.00	0.01
7	Cercozoa	0.00	0.00	Rozellomycota	0.00	0.00
Total		100.00			100.00	

[a]Percentage of relative abundance (mean of 6 samples)
[b]Standard deviation of the mean
*Indicate significant difference

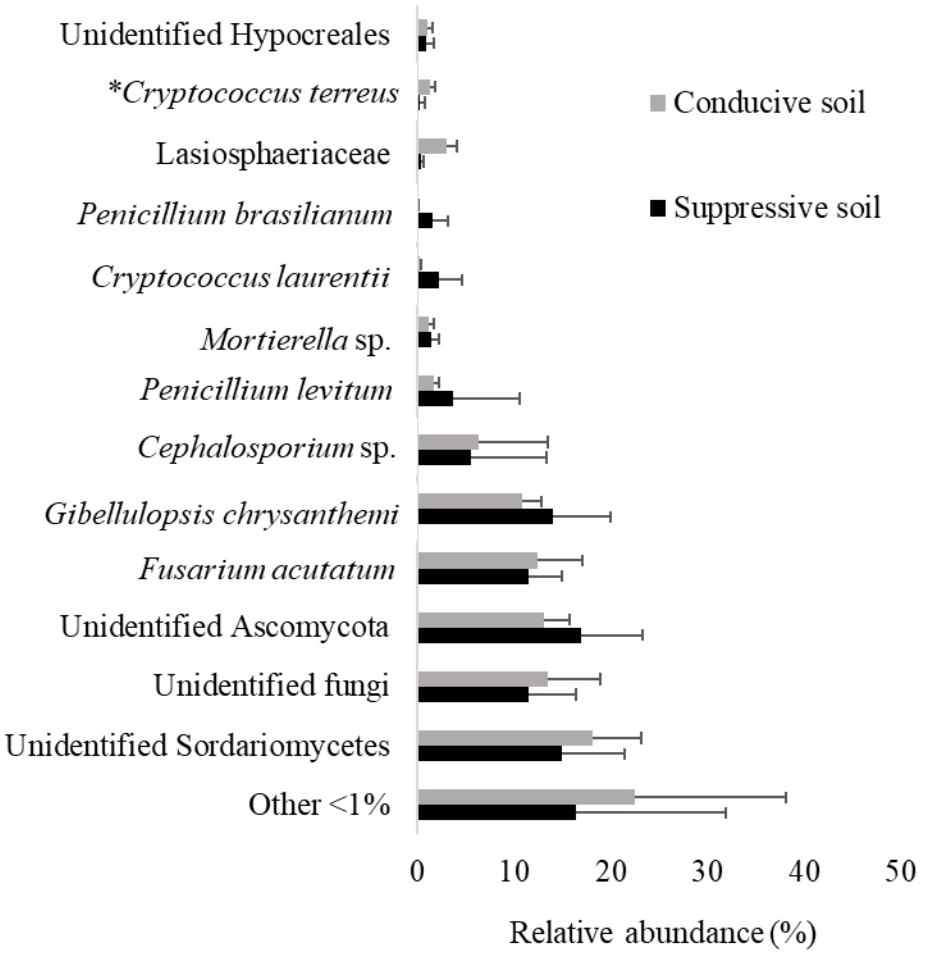

Figure 16. Relative abundance of the fungal species found in suppressive and conducive soil. * The relative abundance is significantly different between suppressive and conducive soil, based on a Welch's t-test analysis. Bars are standard deviation of the mean (n=6).

Table 16. Significant differences in the relative abundance of fungal species (%) ± standard error of the mean (n=6) between suppressive and conducive soils to *Pythium tracheiphilum*, and possible functions of species belonging to the same genus reported in the literature.

Phylum	Family	Genera	Species[a]	Soil class		Increase or decrease[b]	*p*-value	Possible functions (reference)
				Suppressive	Conducive			
Ascomycota	Hyaloscyphaceae	*Arachnopeziza*	*obtusipila*	0.022± 0.019	0.141± 0.068	6.4x	0.01	/
	Pleosporaceae	*Ulocladium*	***chartarum***	0.005± 0.007	0.044± 0.031	8.2x	0.04	Plant pathogen (Zarandi and Sharzei 2015)
Basidiomycota	Cystofilobasidiaceae	*Guehomyces*	*pullulans*	0.256± 0.153	0.9± 0.513	3.5x	0.04	/
	Leucosporidiaceae	*Leucosporidium*	*drummii*	0.092± 0.069	0.019± 0.008	8.6x	0.05	Biocontrol (Vero et al. 2013)
	Tremellales*	*Cryptococcus*	***terreus***	0.309± 0.436	1.301± 0.513	4.2x	0.01	Biocontrol (Wiyono and Agustina 2013)

[a] In bold when the reference involved the species. Not in bold when the reference involved another species from the same genus

[b] Underlined when the relative abundance is lower in the suppressive soil

*Incertae sedis family

Table 17. Fungal species specific to either conducive or suppressive soil to *Pythium tracheiphilum*. Expressed in relative abundance (%) ± standard deviation (n=6), and possible functions of species belonging to the same genus reported in the literature.

				Soil class		
Phylum	**Family**	**Genera**	**Species**[a]	**Suppressive**	**Conducive**	**Possible functions (reference)**
Ascomycota	Chaetomiaceae	*Chaetomium*	*carinthiacum*	0	0.001± 0.001	Biocontrol (Zhao et al. 2017)
		Mycothermus	*thermophilus*	0.009± 0.02	0	/
	Clavicipitaceae	*Pochonia*	***bulbilosa***	0.004± 0.005	0	Nemathophagus (Nicola et al. 2014)
	Helotiaceae	*Rhizoscyphus*	*sp*	0.008± 0.006	0	Ericoid mycorrhiza (Bruzone et al. 2016)
	Microascaceae	*Microascus*	*brevicaulis*	0	0.011± 0.014	Biocontrol (XiaoJia et al. 2010)
	Myxotrichaceae	*Oidiodendron*	*rhodogenum*	0.006± 0.006	0	Ericoid mycorrhiza (Dalpé 1986)
	Onygenaceae	*Chrysosporium*	*sp*	0	0.275± 0.22	/
	Pezizomycotina*	*Ochronis*	*tshawytschae*	0	0.001± 0.002	/
		Remersonia	*thermophila*	0	0.044± 0.032	/
	Pleosporales*	*Stagonosporopsis*	*crystalliniformis*	0.009± 0.009	0	/
	Saccaromycetales*	*Candida*	*subhashii*	0.005± 0.006	0	Biocontrol (Abadias et al. 2005; Lahlali and Jijakli 2009; Lemos et al. 2016; McGuire 1994)
		Nadsonia	*starkeyi-henricii*	0.002± 0.003	0	/
	Trichocomaceae	*Aspergillus*	*austroafricanus*	0	0.016± 0.021	/

		Aspergillus	*pseudodeflectus*	0	0.077± 0.063	/
		Byssochlamys	***spectabilis***	0.035± 0.066	0	Endophyte, biocontrol (Rodrigo et al. 2017)
		Byssochlamys	*zollerniae*	0.152± 0.146	0	Biocontrol (Park et al. 2001)
		Penicillium	*chrysogenum*	0	0.144± 0.105	
		Talaromyces	*sp*	0.015± 0.019	0	Growth stimulator, biocontrol (Naraghi et al. 2012; Naraghi et al. 2010)
	Trichomonascaceae	*Blastobotrys*	*mokoenaii*	0.035± 0.034	0	Biocontrol (Papasotiriou et al. 2013)
	Unidentified	*Leotiomycete*	*sp*	0.01± 0.015	0	/
Basidiomycota	Trichosporonaceae	*Trichosporon*	*dehoogii*	0.015± 0.129	0	/
	Urocystidaceae	*Vankya*	*heufleri*	0.006± 0.003	0	/
	Ustilaginaceae	*Ustilago*	***maydis***	0	0.003± 0.002	Plant pathogen (corn smut)
Chrytridiomycota	Rhizophyctidaceae	*Rhizophlyctis*	*rosea*	0.085± 0.181	0	/
Zygomycota	Umbelopsidaceae	*Umbelopsis*	*sp*	0.002± 0.002	0	/

[a]In bold when the reference involved the species. Not in bold when the reference involved another species from the same genus

* incertae sedis family

4.5 Discussion

In the Naperville county (Quebec, Canada), Pythium stunt (*Pythium tracheiphilum*) is responsible for great losses in head lettuce production. The control of the disease in this area is mainly based on the use of chemical fungicides that are applied preventively all over the growing season. However, it is well known that the use of fungicides over time is responsible for the reduction of the soil life diversity in addition to the economic costs such practices may imply (Chellemi et al. 2016). In recent years, many studies have focused on the suppressive character of certain soils which were found to suppress the pathogenic activity of plant pathogenic species by the interaction with other soil microorganisms (Gomez Exposito et al. 2017; Mazzola and Freilich 2017; Trivedi et al. 2017). Indeed, the assessment of natural soil suppressiveness toward *P. tracheiphilum* by the use of metagenomic analysis, may provide new knowledge necessary in the development of an integrated pest management strategy.

In this study, unexpectedly, bacterial, eukaryotic or fungal composition was not different when diseased and healthy plants were compared. These results contrast with those found by Wu et al. (2015) as well as by Filion et al. (2004), where microbial communities were significantly different between healthy and diseased plants (*Panax notoginseng* or *Picea mariana*) affected by root rot complex. However, when the suppressive and conducive soil were compared, significant differences in the microbial diversity were observed. This result may suggest that disease suppression related to biological aspect is more likely to be observed at a larger scale than at a small scale (within the same small experimental plot). Small-scale spatial variation of *P. tracheiphilum* inoculum density within the experimental plots could possibly explain why one plant was

affected whereas the neighbouring plant was not. However, small-scale spatial variation of inoculum density for bulked soil samples has not been investigated thoroughly. Comparison between diseased and healthy plants at one-site scale could also have been interesting to do in order to validate the similarity or dissimilarity between microbial composition.

In this study, Actinobacteria and Proteobacteria were the two most abundant phyla for both suppressive and conducive classes, followed by Chloroflexi, Firmicutes and Acidobacteria but in a different order of predominance. Predominance of those taxa within the rhizosphere of lettuce plants has also been observed in another study. Cardinale et al. (2015) found that the rhizosphere of different lettuce cultivars was dominated, in strict order, by members of Proteobacteria, Bacterioidetes and in a lesser extent by Chloroflexi and Actinobacteria.

For bacteria and eukaryotes, microbial composition was different between SS and CS classes. However, neither the alpha-diversity estimation (Chao1 index) nor the evenness estimation (Shannon index) differed between classes. Difference in the bacterial composition could possibly be explained by the greater abundance of Chloroflexi and Gemmatiomonadetes in the CS samples. Differences in eukaryotes composition remains however difficult to explain by the relative abundance of certain taxa since none of the most abundant taxa were significantly higher in one class compared to the other.

At a deeper taxonomic level, the bacterial genus *Opitutus* was found in higher abundance in SS samples than in CS samples. Interestingly, the Opitutacea family was characterized as plant-beneficial bacteria that have antagonistic activities toward pathogens and was associated with healthy cotton plants (Li et al. 2015). Moreover, the genus

Mucilaginibacter was found to be specific to SS class since it was not recovered in any CS samples. According to Madhaiyan et al. (2010), two species of the *Mucilaginibacter* genus, *M. gossypii* and *M. gossypiicola,* were recovered in cotton rhizosphere soil and were defined as plant-growth-promoting bacteria for cotton plant.

For eukaryotes, the Phytomyxea family was found in higher abundance in the SS class than in the CS class. Members of Phytomyxea are known to be parasite of Pythium species. *Woronina pythii,* demonstrated hyper-parasitic activities on vegetative hyphae and reproductive structures of seven different *Pythium* species including, *P. aphanidermatum, P. irregulare,* and *P. ultimum* (Dylewski and Miller 1984). Similarly, *Sorodiscus cokeri* showed parasitic activities toward six different *Pythium* species including *P. graminicolum, P. catenulatum,* and *P. irregulare* (Goldie-Smith 1951). However, in this study, only the genus *Spongospora sp* could be identified, the remaining representative of the Phytomyxea family being other or uncultured genera, and nothing is mentioned in the literature concerning potential biocontrol activities of *Spongospora* species. In the conducive soil, the nematode *Fridericia tuberosa* was found in higher abundance. The role of meso- or microfauna in plant disease suppression is not or poorly described in the literature, but their herbivorous activity may favor the infection of plant-pathogen (Kamal and McSpadden Gardener 2006).

In this study, the fungal composition was not significantly different when SS and CS classes were compared. However, Shannon index was significantly greater for the CS class than for SS class, suggesting that the OTUs were more evenly distributed in the conducive soil than in the suppressive soil. On the other hand, Chao1 index was not different between the classes, which indicates that the number of different OTUs was

similar between the classes. The facts that first, Shannon index was greater in the CS class and second, that many fungal species or genera were found to be specific to the one or the other classes may, however, raise questions concerning the similarity of the fungal composition obtained with the Permanova analysis.

Relative abundance of the Basidiomycota was significantly higher in the suppressive than in the conducive soil in this study and was dominated by the genus *Cryptococcus*, especially the species *C. laurentii* and *C. terreus.* Several *Cryptococcus* species including *C. laurentii* are known for their biocontrol potential in the literature (Fan and Tian 2001; Rong and McSpadden Gardener 2013). *C. laurentii* is known to control the pathogen *Colletotrichum gloeosporioides* in mango trees, and *Penicillium expansum* in jujube fruit and other plant species (Bautista-Rosales et al. 2014; Cao et al. 2012).

At deeper taxonomic level, the rare species *Leucosporidium drummii* was the only fungal species found in higher abundance in the SS than in the CS class. This species was not reported in the literature, however, *L. scotti* was identified as a good biocontrol agent against blue (*Penicillium expansum*) and gray (*Botrytis cinerea*) molds of apple (Vero et al. 2013). In this study, 17 fungal species or genera were found to be specific to the SS class, while only 10 were specific to the CS class. Some of those species that were specific to SS class, or some representative of the same genus, were found to be of interest in the literature. First, the endophytes *Bissochlamys spectabilis* (anamorph *Paecilomyces variotii*) as well as *B. zollerniae* were specific to suppressive soil in this study. *Bissochlamys spectabilis* was identified as a potential biocontrol agent against pathogenic *Fusarium moniliforme*, when evaluated on *Lolium rigidium* (Rodrigo et al. 2017). Another *Byssochlamys* species, *B. nivea*, was also found to inhibit, *in vitro*, the growth of four plant-

pathogenic fungi: *Gaeumannomyces graminis*, *Phytophtora cinnamomi*, *Fusarium oxysporum* and *Rhizoctonia solani* (Park et al. 2001). Second, the Saccharomycete *Blastobotrys mokoenaii* was also specific to suppressive soil samples. Unidentified species (strain FP12) of the *Blastrobotrys* genus was found to reduce the microsclerotia germination of the ascomycete *Verticillium dahliae* and reduced wilt severity in eggplants (Papasotiriou et al. 2013). Unidentified species of *Talaromyces* was also specific to suppressive soil in the present study. The species *T. flavus*, was found to stimulate the growth of cotton and potato plants (Naraghi et al. 2012) and to be an antagonist of the ascomycete *Verticillium albo-atrum*, the responsible agent of cucumber wilt in greenhouses (Naraghi et al. 2010). Saccharomycetes *Candida subhashii* was also specific to suppressive soil. This species is not mentioned in the literature to be of interest as a biocontrol. However, four other species of the same genus were found to be: *C. oleophila* and *C. sake* against *Penicillium expansum* (blue mold of apple) (Abadias et al. 2005; Lahlali and Jijakli 2009), *C. guilliermondhii* against *Penicillium digitatum* in grapefruit (McGuire 1994) and *C. zemplinina* against *Botrytis cinerea* in grapes (Lemos et al. 2016). The Sordariomycete *Pochonia bulbilosa* was also recovered only in suppressive soil samples. This species, as well as another species of the same genus, *P. chlamydospora*, are known to be antagonist of plant-parasitic nematodes, including species from the *Meloidogyne* genus (Manzanilla-López et al. 2013; Mukhtar et al. 2013; Nicola et al. 2014; Viggiano et al. 2014). The presence of *Nematophagus* species in the context of soil suppressiveness to *P. tracheiphilum* could be interesting since example of interaction between microfauna and plant-pathogen has already been investigated as it is the case for the nematode *Meloidogyne hapla* and *P. tracheiphilum*, for which their simultaneous

presence in a soil grown with lettuces shown an additive effect in the reduction of the plant growth (Gracia et al. 1991).

At deeper taxonomic level, the rare fungal species *Ulocladium chartarum* as well as *Cryptococcus terreus* were found in higher abundance in the conducive soil than in the suppressive soil. *Ulocladium chartarum* is known to cause leaf spot in lemon verben (Zarandi and Sharzei 2015) and to be genetically close-related to plant pathogenic *Alternaria* sp and *Stemphylium* sp. (Pryor and Gilberston 2000). *Cryptococcus terreus* however, was found to be a yeast antagonist of *Curvularia pallescens* causing the petal blight of *Dendrobium* sp. In this study, 10 species were found to be specific to conducive soil. From those, there is the plant pathogen *Ustilago maydis* the agent of corn smut, *Microascus brevicaulis* (synonym *Scopulariopsis brevicaulis*), which was found to be antagonist of *Sclerotinia sclerotiorum* by inhibiting sclerotial germination (XiaoJia et al. 2010), and *Chaetomium carinthiacum* for which another species of the same genus, the endophyte *C. globosum* was found to be a potential biocontrol agent against the same pathogen (Zhao et al. 2017).

In summary, in the suppressive soil, nine different taxa (either bacteria, eukaryote or fungi) recovered only or in higher abundance in the suppressive soil were reported in the literature to act as plant-beneficial organisms or biocontrol agents, notably the genus *Opitutus* (Verrucromicrobia), the class of Phytomyxea (eukaryote) and *Leucosporidium drummii* (Basidiomycota). In contrast, in the conducive soil, only three taxa were mentioned in the literature, and more plant pathogens were recovered. Moreover, more specific fungal species were recovered in the suppressive soil than in the conducive soil, suggesting a protective effect of a higher microbial diversity of rare species present in the

rhizosphere. This assumption is consistent with results obtain by Cardinale et al. (2015). One of their conclusion, when evaluating the bacterial networks and relationships in the lettuce root microbiota, was that the combination of the presence of beneficial rare species along with a high diversity could increase the protection against plant pathogens. Results of the present study suggests that pathogenicity of *P. tracheiphilum* is modulated by the microbial community naturally inhabiting the root zone and highlighted potential biological characteristic of a soil suppressive to *P. tracheiphilum*. The management of the indigenous soil microbiome for biological control has been subject to investigation in the recent years, and according to Mazzola and Freilich (2017), it should provide a more sustainable means to control soilborne diseases relative to the simple introduction of a non-native biocontrol agent. Enhancement of natural soil suppressiveness is mainly based on the addition of substrates with the capacity to modify soil's microbial community in the disfavor of plant pathogen. For instance, the addition of chitin to the soil has been proven to increase disease suppressiveness against *Verticillium dahliae* and *Rhizoctonia solani* in lettuce plants by increasing the relative abundance of several bacterial and fungal phyla including Verrucomicrobia, and Basidiomycota, while increasing the fresh weight of lettuce as well (Debode et al. 2016). Hence, further investigation on cultural practices or organic amendment promoting natural suppression of *P. tracheiphilum* in lettuce grown in the soil of the Napierville county would be an interesting next step to accomplish.

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5. General conclusions and consideration for future research

Relationship between inoculum density and disease incidence is becoming increasingly studied for soilborne pathogens, mainly because of the development of molecular tools such as real time PCR, which greatly facilitate the work. In the first part of this study, the author intended to define the relationship between soilborne inoculum of *P. tracheiphilum*, weather parameters and incidence of Pythium stunt under head lettuce field conditions. Results obtained from this study strongly suggest the possibility of using soil inoculum density, air temperature and rain accumulation to accurately predict the disease. This new knowledge therefore raises the possibility of using those predictors as a decision tool to guide the lettuce producers during planning for their cropping. However, in this study, the within-field spatial distribution of soil inoculum, the cultivars effect as well as the variation in the virulence of *P. tracheiphilum* community among fields has not been investigated. It would therefore be necessary for future research to address such issues before the implementation of a decision tool at a larger scale.

In the second part of this study, using metagenomic analysis, the soil rhizosphere of lettuces grown in either a suppressive or a conducive soil to *P. tracheiphilum* was characterized in order to know if the native microbial community of the soil could modulate the pathogenicity of *P. tracheiphilum*, and if so, to decipher which biological features are involved in disease suppression. Results from this study suggest that a higher abundance of Basidiomycota as well as the presence of more plant-beneficial or pathogen-antagonistic rare species, either bacterial, eukaryotic or fungal, are associated with a suppressive soil to *P. tracheiphilum*. However, since this second part of the study was conducted in an

explanatory manner, biological differences found between the conducive and suppressive soil absolutely need to be validate with a larger dataset. In the prospect of future research, to characterize a suppressive soil to *P. tracheiphilum* prior to planting instead of the soil from the plant rhizosphere would be also interesting. Because the microbial community around the plants is strongly influenced by the plant system, that information cannot be used to predict the suppressive character of a soil prior to crop establishment, thus limiting the practical utilization of such knowledge for the development of decision tool. Biological characterization of bulk soil to identify microbial predictors for soil suppressiveness has recently given good results in Australia (Trivedi et al. 2017), thus demonstrating the relevance of continuing in this perspective.

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6. Literature cited

Abadias, M., Teixidó, N., Usall, J., Solsona, C., and Viñas, I. 2005. Survival of the postharvest biocontrol yeast *Candida sake* CPA-1 after dehydration by spray-drying. Biocontrol Science and Technology 15:835-846.

AL-Hamdani, A. M., Lutchmeah, R. S., and Cooke, R. C. 1983. Biological control of *Pythium ultimum*-induced damping-off by treating cress seed with the mycoparasite *Pythium oligandrum*. Plant Pathology 32:449-454.

Alhussaen, K. M. 2012. Effect of Soil Acidity on Diseases Caused by *Pythium ultimum* and *Fusarium oxysporum* on Tomato Plants. Journal of Biological Sciences 12:416-420.

Almquist, C., Persson, L., Olsson, Å., Sundström, J., and Jonsson, A. 2016. Disease risk assessment of sugar beet root rot using quantitative real-time PCR analysis of *Aphanomyces cochlioides* in naturally infested soil samples. European Journal of Plant Pathology 145:731-742.

Baker, R. 1978. Inoculum potential. Pages 135-157 in: Plant Disease: An advanced Treatise, vol. II. How Disease Develops in Populations. Academic Press, New York.

Bardin, S., Huang, H. C., Pinto, J., Amundsen, E. J., and Erickson, R. S. 2004. Biological control of Pythium damping-off of pea and sugar beet by *Rhizobium leguminosarum* bv. *Viceae*. Revue canadienne de botanique 82:29-296.

Barr, D. J. S., Warwick, S. I., and Désaulniers, N. L. 1997. Isozyme variation in heterothallic species and related asexual isolates of Pythium. Canadian Journal of Botany 75:1927-1935.

Bautista-Rosales, P. U., Calderon-Santoyo, M., Servín-Villegas, R., Ochoa-Álvarez, N. A., Vázquez-Juárez, R., and Ragazzo-Sánchez, J. A. 2014. Biocontrol action mechanisms of *Cryptococcus laurentii* on *Colletotrichum gloeosporioides* of mango. Crop Protection 65:194-201.

Baysal-Gurel, F. and Miller, S. 2010. Disease management in organic lettuce production. in: Organic Agriculture. http://articles.extension.org/pages/30849/disease-management-in-organic-lettuce-production. [Accessed 2018]

Berbegal, M., Álvarez, L. A., Pérez-Sierra, A., and Armengol, J. 2011. Identification of *Pythium tracheiphilum* as the causal agent of vascular necrosis of endive (Cichorium endivia) in Spain. Phytoparasitica 40:1-4.

Biesbrock, J. A., and Hendrix, F. F. 1970. Influence of soil water and temperature on root necrosis of peach caused by *Pythium* spp. Phytopathology 82:880-882.

Blancard, D., Lot, H., and Maisonneuve, B. 2003. Diseases of lettuces: identification, detection and control. INRA Editions, Paris.

Blancard, D., Lot, H., and Maisonneuve, B. 2005. A color Atlas of diseases of lettuce and related salad crops: observations, biology and control. Manson Publising Ltd., London.

Blok, I., and Plaats-Niterink, A. J. 1978. *Pythium uncinulatum* sp. nov. and *P. tracheiphilum* pathogenic to lettuce. Netherlands Journal of Plant Pathology 84:135-147.

Boehm, M. J., Wu, T., Stone, A. G., Kraakman, B., Iannotti, D. A., Wilson, G. E., Madden, L. V., and Hoitink, H. A. J. 1997. Cross-polarized magic-angle spinning 13C nuclear magnetic resonance spectroscopic characterization of soil organic matter relative to culturable bacterial species composition and sustained biological control of pythium root rot. Applied and Environmental Microbiology 63:162-168.

Boughalleb, N., and El Mahjoub, M. 2006. *Fusarium solani* f.sp. cucurbitae and *F. oxysporum* f.sp. *niveum* inoculum densities in Tunisian soils and their effect on watermelon seedlings. Phytoparasitica 34:149-158.

Bratoloveanu, J., and Wallace, H. R. 1985. The influence of *Pythium* on the growth of barley seedlings as affected by soil water and inoculum density. Plant and Soil 85:305-3011.

Brown, G. E., and Kennedy, B. W. 1966. Effect of oxygen concentration on *Pythium* seed rot of soybean. Phytopathology 56:407-411.

Bruggen, A. H. C. v., and Semenov, A. M. 2000. In search of biological indicators for soil health and disease suppression. Applied Soil Ecology 15:13-24.

Bruzone, M., Fehrer, J., B Fontenla, S., and Vohník, M. 2016. First record of *Rhizoscyphus ericae* in Southern Hemisphere's Ericaceae.

Campbell, G. S., and Neher, D. A. 1994. Estimating disease severity and incidence. Pages 117-147 in: Epidemiology and management of root diseases. C. L. Campbell, ed.

Campoverde, E. V., Sanahuja, G., and Palmateer, A. J. 2017. A High Incidence of *Pythium* and *Phytophthora* Diseases Related to Record-breaking Rainfall in South Florida. HortTechnology 27:78-83.

Cao, B., Li, H., Tian, S., and Qin, G. 2012. Boron improves the biocontrol activity of *Cryptococcus laurentii* against *Penicillium expansum* in jujube fruit. Postharvest Biology and Technology 68:16-21.

Cardinale, M., Grube, M., Erlacher, A., Quehenberger, J., and Berg, G. 2015. Bacterial networks and co-occurrence relationships in the lettuce root microbiota. Environmental Microbiology 17:239-252.

Chapelle, E., Mendes, R., Bakker, P. A., and Raaijmakers, J. M. 2016. Fungal invasion of the rhizosphere microbiome. Journal of the International Society for Microbial Ecology 10:265-268.

Chellemi, D. O., Gamliel, A., Katan, J., and Subbarao, K. V. 2016. Development and deployment of systems-based approaches for the management of soilborne plant pathogens. Phytopathology 106:216-225.

Chen, M.-H., Jack, A. L. H., McGuire, I. C., and Nelson, E. B. 2012. Seed-colonizing bacterial communities associated with the suppression of pythium seedling disease in a municipal biosolids compost. Phytopathology 102:478-489.

Cherif, M., Tirilly, Y., and Belanger, R. R. 1997. Effect of oxygen concentration on plant growth, lipidperoxidation, and receptivity of tomato roots to *Pythium* F under hydroponic conditions. European Journal of Plant Pathology 103:255-264.

Chet, I. 1981. *Trichoderma* - application, mode of action, and potential as a biocontrol agent of soil-borne plant pathogen. Pages 137-160 in: Innovative Approaches to Plant Disease Control. I. Chet, ed. Wiley-Interscience; 99 edition, New York.

Chet, I., Harman, G. E., and Baker, R. 1981. *Trichoderma hamatum*: Its hyphal interactions with *Rhizoctonia solani* and *Pythium* spp. Microbial Ecology 7:29-38.

Comeau, A. M., Li, W. K. W., Tremblay, J.-É., Carmack, E. C., and Lovejoy, C. 2011. Arctic ocean microbial community structure before and after the 2007 record sea ice minimum. PLoS ONE 6:e27492.

Consortium PRISME. 2016. LAITUE: Ressource pour producteurs, conseillers et étudiants. https://prisme.ca/documentation/laitue/. [Accessed March 2018]

CRAAQ, centre de référence en agriculture et agroalimentaire du Québec. 2011. Guide de référence en fertilisation. 2e édition ed.

CRAAQ SAgE pesticide. 2017. Traitements phytosanitaires et risques associés. https://www.sagepesticide.qc.ca. [Accessed January 2018]

Cullen, D. W., Lees, A. K., Toth, I. K., and Duncan, J. M. 2001. Conventional PCR and real-time quantification PCR detection of *Helminthosporium solani* in soil and on potato tubers. European Journal of Plant Pathology 107:387-398.

Cullen, D. W., Lees, A. K., Toth, I. K., and Duncan, J. M. 2002. Detection of *Colletotrichum coccodes* from soil and potato tubers by conventional and quantitative real-time PCR. Plant Pathology 51:281-292.

Cullen, D. W., Toth, I. K., Boonham, N., Walsh, K., Barker, I., and Lees, A. K. 2007. Development and Validation of Conventional and Quantitative Polymerase Chain Reaction Assays for the Detection of Storage Rot Potato Pathogens, *Phytophthora erythroseptica*, *Pythium ultimum* and *Phoma foveata*. Journal of Phytopathology 155:309-315.

Dalpé, Y. 1986. Axenic synthesis of ericoid mycorrhiza in *Vaccinium augustifolium* Ait. by *Oidiodendron* species. New Phytologist 103:391-396.

Debode, J., De Tender, C., Soltaninejad, S., Van Malderghem, C., Haegeman, A., Van der Linden, I., Cottyn, B., Heyndrickx, M., and Maes, M. 2016. Chitin Mixed in Potting Soil Alters Lettuce Growth, the Survival of Zoonotic Bacteria on the Leaves and Associated Rhizosphere Microbiology. Front Microbiol 7:565.

DeVay, J. E., Garber, R. H., and Matheron, D. 1982. Role of *Pythium* species in the seedling disease complex of cotton in california. Plant Disease 66:151-154.

Dick, M. W. 1990. Keys to *Pythium*. Reading, UK.

Dick, M. W., and Ali-Shtayeh, M. S. 1986. Distribution and frequency of *Pythium* species in parkland and farmland soils. Transactions of the British Mycological Society 86:49-62.

Dylewski, D. P., and Miller, C. E. 1984. The ultrastructure of mitosis during sporangiogenesis in *Woronina pythii* (Plasmodiophoromycetes). Protoplasma 121:42-53.

El-Tarabily, K. A., Hardy, G. E. S. J., and Sivasithamparam, K. 2010. Performance of three endophytic actinomycetes in relation to plant growth promotion and biological control of *Pythium aphanidermatum*, a pathogen of cucumber under commercial field production conditions in the United Arab Emirates. European Journal of Plant Pathology 128:527-539.

Erlacher, A., Cardinale, M., Grube, M., and Berg, G. 2015. Biotic stress shifted structure and abundance of Enterobacteriaceae in the lettuce microbiome. PLoS One 10:e0118068.

Eyre, C. A., and Garbelotto, M. 2015. Detection, diversity, and population dynamics of waterborne *Phytophthora ramorum* populations. Phytopathology 105:57-68.

Fall, M. L., Van der Heyden, H., Beaulieu, C., and Carisse, O. 2015. *Bremia lactucae* infection efficiency in lettuce is modulated by temperature and leaf wetness duration ender Quebec field conditions. Plant Disease 99:1010-1019.

Fan, Q., and Tian, S. P. 2001. Postharvest biological control of grey mold and blue mold on apple by *Cryptococcus albidus* (Saito) Skinner. Postharvest Biology and Technology 21:341-350.

Filion, M., Hamelin, R. C., Bernier, L., and St-Arnaud, M. 2004. Molecular profiling of rhizosphere microbial communities associated with healthy and diseased black spruce (*Picea mariana*) seedlings grown in a nursery. Applied and Environmental Microbiology 70:3541-3551.

Gangneux, C., Cannesan, M.-A., Bressan, M., Castel, L., Moussart, A., Vicré-Gibouin, M., Driouich, A., Trinsoutrot-Gattin, I., and Laval, K. 2014. A sensitive assay for rapid detection and quantification of *Aphanomyces euteiches* in soil. Phytopathology 104:1138-1147.

Gilles, T., Phelps, K., Clarkson, J. P., and Kennedy, R. 2004. Development of MILIONCAST, an improved model for predicting downy mildew sporulation on onions. Plant Disease 88:695-702.

Goldie-Smith, E. K. 1951. A new species of *Sorodiscus* on *Pythium*. Journal of the Elisha Mitchell Scientific Society 67:108–121.

Gomez Exposito, R., de Bruijn, I., Postma, J., and Raaijmakers, J. M. 2017. Current Insights into the Role of Rhizosphere Bacteria in Disease Suppressive Soils. Frontiers in Microbiology 8:2529.

Gonzalez, A. J., Tello, J. C., and Herrero, M. L. 2004. First report of *Pythium tracheiphilum* causing wilt and leaf blight on lettuce (*Lactuca sativa*) in Spain. Plant disease 88:1382.

Gracia, J. A. 1989. Lettuce stunt : effect of *Pythium* populations and interactions between *Pythium tracheiphilum* and nematodes. Thesis (M Sc).

Gracia, J. A., Reeleder, R. D., and Bélair, G. 1991. Interactions between *Pythium tracheiphilum*, *Meloidogyne hapla* and *Pratylenchus penetrans* on lettuce. Phytoprotection 72:105.

Griffin, D. M. 1958. Influence of pH on the incidence of damping-off. Transactions of the British Mycological Society 41:483-490.

Gutierrez, W. A., and Melton, T. A. 2001. Pathogen structures. in: Pythium Root Rot in Tobacco Greenhouses, Tobacco disease note No.8 North Carolina State University.

Hancock, J. G. 1991. Seedling and rootlet diseases of forage alfalfa caused by *Pythium irregulare*. Plant Disease 75:691-694.

Harman, G. E., Taylor, A. G., and Stasz, T. E. 2004. Combining Effective Strains of *Trichoderma harzianum* and Solid Matrix Priming to Improve Biological Seed Treatments. Plant Disease 73:631-637.

Hayden, K. J., Rizzo, D., Tse, J., and Garbelotto, M. 2004. Detection and quantification of Phytophthora ramorum from California forests using a real-time polymerase chain reaction assay. Phytopathology 94:1075-1083.

Heffer Link, V., Powelson, M. L., and Johnson, K. B. 2002. Oomycetes. in: The Plant Health Instructor. https://www.apsnet.org/edcenter/intropp/LabExercises/Pages/Oomycetes.aspx. [Accessed 2018].

Höper, H., Steinberg, C., and Alabouvette, C. 1995. Involvement of clay type and pH in the mechanisms of soil suppressiveness to Fusarium wilt of flax. Soil Biology and Biochemistry 27:955-967.

Hoppe, P. E. 1966. *Pythium* species still viable after 12 years in ai dried muck soil. Phytopathology 56:1411.

Howell, C. R. 2002. Cotton seedling preemergence damping-off incited by *Rhizopus oryzae* and *Pythium* spp. and its biological control with *Trichoderma* spp. Phytopathology 92:177-180.

ISQ, Institut de la statistique du Québec, and MAPAQ, Ministère de l'Agriculture des Pêcheries et de l'Alimentation du Québec 2016. Profil sectoriel de l'industrie horticole au Québec.

Jacquet, M. 1979. *Pythium tracheiphilum* Matta, agent du flétrissement de la laitue (*Lactuca Sativa* L.): Étude biologique et épidémiologique. Ph.D. Université des Sciences et Techniques de Lille.

Jeger, M. J, and Termorshuizen, A. J. 2012. The theory of inoculum: the relationship between disease incidence and inoculum density and dynamics of soilborne plant pathogens. Pages 3-15 in: Soil Solarization: Theory and Practice. A. Gamliel and J. Katan, eds. The American Phytopathology Society, St.Paul, MN.

Kamal, K. P. and McSpadden Gardener, B. 2006. Biological Control of Plant Pathogens in: The Plant Health Instructor. https://www.apsnet.org/edcenter/advanced/topics/Documents/PHI-BiologicalControl.pdf. [Accessed 2018]

Katan, J. 2017. Diseases caused by soilborne pathogens: biology, management and challenges. Journal of Plant Pathology 99:305-315.

Katan, J., Shtienberg, D. and Gamliel, A. 2012. The integrated management concepts in the context of soilborne pathogens and soil disinfestation. Pages 91-97 in: Soil Solarization: Theory and Practice. A. Gamliel and J. Katan, eds. The American Phytopathology Society, St.Paul, MN.

Keinath, A. P. 1995. Relationships between inoculum density of *Rhizoctonia solani*, wirestem incidence and severity, and growth of cabbage. Phytopathology 85:1487-1492.

Kenaga, C. B., Williams, E. B., and Green, R. J. 1971. Plant Disease Syllabus. Balt Publishers.

Kernaghan, G., Reeleder, R. D., and Hoke, S. M. T. 2008. Quantification of *Pythium* populations in ginseng soils by culture dependent and real-time PCR methods. Applied Soil Ecology 40:447-455.

Kiehr, M., Delhey, R., and Lusto, J. 2015. Wilt and vascular root rot (*Pythium tracheiphilum*) of lettuce in Bahía Blanca, Argentina. Phyton 84:423-426.

Kipngeno, P., Turoop, L., Maina, N., Kahangi, E., and Juma, P. 2015. Efficacy of *Bacillus Subtilis* and *Trichoderma asperellum* against *Pythium aphanidermatum* in tomatoes.

Klein, E., Ofek, M., Katan, J., Minz, D., and Gamliel, A. 2013. Soil suppressiveness to *Fusarium* disease: Shifts in root microbiome associated with reduction of pathogen root colonization. Phytopathology 103:23-33.

Kleinhenz, B., Falke, K., Kakau, J., and Rossberg, D. 2007. SIMBLIGHT1 a new model to predict first occurrence of potato late blight. EPPO Bulletin 37:339-343.

Klemsdal, S. S., Herrero, M. L., Wanner, L. A., Lund, G., and Hermansen, A. 2008. PCR-based identification of *Pythium* spp. causing cavity spot in carrots and sensitive detection in soil samples. Plant Pathology 57:877-886.

Kogan, M. 1998. Integrated pest management: Historical perspectives and contemporary developments. Annual Review of Entomology 43:243-270.

Kõljalg, U., Nilsson, R. H., Abarenkov, K., Tedersoo, L., Taylor, A. F. S., Bahram, M., Bates, S. T., Bruns, T. D., Bengtsson-Palme, J., Callaghan, T. M., Douglas, B., Drenkhan, T., Eberhardt, U., Dueñas, M. l., Grebenc, T., Griffith, G. W., Hartmann, M., Kirk, P. M., Kohout, P., Larsson, E., Lindahl, B. D., Lücking, R., Martín, M. P., and Matheny, P. B. 2013. Towards a unified paradigm for sequence-based identification of fungi. Molecular Ecology 22:5271-5277.

Kouyeas, V. 1964. An approach to the study of moisture relations of soil fungi. Plant and Soil 20:351-363.

Kumar, S., Ray, J., Davison, E. M., Cunnington, J. H., and S., A. d. 2007. First record of *Pythium tracheiphilum* associated with lettuce wilt and leaf blight in Australia. Australasian Plant Disease Notes 2:7-9.

Kushalappa, A. C. 2001. BREMCAST: Development of a system to forecast risk levels of downy mildew on lettuce (*Bremia lactucae*). international journal of pest management 47:1-5.

Lahlali, R., and Jijakli, M. H. 2009. Enhancement of the biocontrol agent *Candida oleophila* (strain O) survival and control efficiency under extreme conditions of water activity and relative humidity. Biological Control 51:403-408.

Lamichhane, J. R., Dürr, C., Schwanck, A. A., Robin, M.-H., Sarthou, J.-P., Cellier, V., Messéan, A., and Aubertot, J.-N. 2017. Integrated management of damping-off diseases. A review. Agronomy for Sustainable Development 37:1-25.

Lamour, K., and Kamoun, S. 2009. Oomycete genetics and genomics: diversity, interactions and research tools. Jon Wiley and Sons.

Lees, A. K., Cullen, D. W., Sullivan, L., and Nicolson, M. J. 2002. Development of conventional and quantitative real-time PCR assays for the detection and identification of *Rhizoctonia solani* AG-3 in potato and soil. Plant Pathology 2002:293-302.

Lemos, W. J. F., Bovo, B., Nadai, C., Crosato, G., Carlot, M., Favaron, F., Giacomini, A., and Corich, V. 2016. Biocontrol ability and action mechanism of *Starmerella bacillaris* (Synonym *Candida zemplinina*) isolated from wine musts against gray mold disease agent *Botrytis cinerea* on grape and their effects on alcoholic fermentation. Frontiers in Microbiology 7: 1-12.

Levesque, C. A., Brouwer, H., Cano, L., Hamilton, J. P., Holt, C., Huitema, E., Raffaele, S., Robideau, G. P., Thines, M., Win, J., Zerillo, M. M., Beakes, G. W., Boore, J. L., Busam, D., Dumas, B., Ferriera, S., Fuerstenberg, S. I., Gachon, C. M., Gaulin, E., Govers, F., Grenville-Briggs, L., Horner, N., Hostetler, J., Jiang, R. H., Johnson, J., Krajaejun, T., Lin, H., Meijer, H. J., Moore, B., Morris, P., Phuntmart, V., Puiu, D., Shetty, J., Stajich, J. E., Tripathy, S., Wawra, S., van West, P., Whitty, B. R., Coutinho, P. M., Henrissat, B., Martin, F., Thomas, P. D., Tyler, B. M., De Vries, R. P., Kamoun, S., Yandell, M., Tisserat, N., and Buell, C. R. 2010. Genome sequence of the necrotrophic plant pathogen *Pythium ultimum* reveals original pathogenicity mechanisms and effector repertoire. Genome Biology 11:R73.

Lévesque, C. A., and De Cock, A. W. A. M. 2004. Molecular phylogeny and taxonomy of the genus *Pythium*. Mycological Research 108:1363-1383.

Li, X., Zhang, T., Zhang, Y., Ding, C., Wang, X., Jia, Z., and He, Z. 2015. Declined soil suppressiveness to *Fusarium oxysporum* by rhizosphere microflora of cotton in soil sickness. Biology and Fertility of Soils 51:935-946.

Lifshitz, R., and Hancock, J. G. 1983. Saprophytic development of *Pythium ultimum* in soil as a function of water matric potential and temperature. Phytopathology 73:257-261.

Littrell, R. H., and McCarter, S. M. 1970. Effect of soil temperature on virulence of *Pythium aphanidermatum* and *Pythium myriotylum* to rye and tomato. Phytopathology 60:704-707.

Madhaiyan, M., Poonguzhali, S., Lee, J. S., Senthilkumar, M., Lee, K. C., and Sundaram, S. 2010. *Mucilaginibacter gossypii* sp. nov. and *Mucilaginibacter gossypiicola* sp. nov., plant-growth-promoting bacteria isolated from cotton rhizosphere soils. International Journal of Systematic and Evolutionary Microbiology 60:2451-2457.

Manzanilla-López, R. H., Esteves, I., Finetti-Sialer, M. M., Hirsch, P. R., Ward, E., Devonshire, J., and Hidalgo-Díaz, L. 2013. *Pochonia chlamydosporia*: Advances and challenges to improve its performance as a biological control agent of sedentary endo-parasitic nematodes. Journal of Nematology 45.

Marie-Victorin, É. C., frères. 1935. Flore Laurentienne. 3th ed.

Martin, F. N., and Loper, J. E. 1999. Soilborne plant diseases caused by *Pythium* spp.: ecology, epidemiology, and prospects for biological control. Critical Reviews in Plant Sciences 18:111-181.

Martinez, C., Levesque, C. A., Belanger, R. R., and Tweddell, R. J. 2005. Evaluation of fungicides for the control of carrot cavity spot. Pest Management Science 61:767-771.

Mazzola, M., and Freilich, S. 2017. Prospects for Biological Soilborne Disease Control: Application of Indigenous Versus Synthetic Microbiomes. Phytopathology 107:256-263.

McGuire, K. L., Payne, S. G., Palmer, M. I., Gillikin, C. M., Keefe, D., Kim, S. J., Gedallovich, S. M., Discenza, J., Rangamannar, R., Koshner, J. A., Massmann, A. L., Orazi, G., Essene, A., Leff, J. W., and Fierer, N. 2013. Digging the New York City Skyline: Soil Fungal Communities in Green Roofs and City Parks. PLoS ONE 8:e58020.

McGuire, R. G. 1994. Application of *Candida guilliermondii* in commercial citrus coatings for biocontrol of *Penicillium digitatum* on grapefruits. Biological Control 4:1-7

Mendes, R., Kruijt, M., de Bruijn, I., Dekkers, E., van der Voort, M., Schneider, J. H. M., Piceno, Y. M., DeSantis, T. Z., Andersen, G. L., Bakker, P. A. H. M., and Raaijmakers, J. M. 2011. Deciphering the Rhizosphere Microbiome for Disease-Suppressive Bacteria. Science 332:1097-1100.

Mendoza, L. n.d. Biology of *Pythium insidiosum*. Michigan State University, Biomedical Laboratory Diagnostics Program. https://bld.natsci.msu.edu/research/pythium-insidiosum/biology-of-pythium/. [Accessed 2016].

Merkle, E. C., and Shaffer, V. A. 2011. Binary recursive partitioning: background, methods, and application to psychology. British Journal of Mathematical and Statistical Psychology 64:161-181.

Milus, E. A., and Rothrock, C. S. 1997. Efficacy of bacterial seed treatments for controlling *Pythium* root rot of winter wheat. Plant Disease 81:180-184.

Moller, K., Jensen, B., Andersen, H. P., Stryhn, H., and Hockenhull, J. 2003. Biocontrol of *Pythium tracheiphilum* in chinese cabbage by *Clonostachys rosea* under field conditions. Biocontrol Science and Technology 13:171-182.

Mukhtar, T., Hussain, M. A., and Kayani, M. Z. 2013. Biocontrol potential of *Pasteuria penetrans*, *Pochonia chlamydosporia*, *Paecilomyces lilacinus* and *Trichoderma harzianum* against *Meloidogyne incognita* in okra. Phytopathologia Mediterranea 52:66-76.

Mündel, H.-H., Huang, H. C., Kozub, G. C., and Barr, D. J. S. 1995. Effect of soil moisture and temperature on seedling emergence and incidence of Pythium damping-off in safflower (*Carthamus tinctorius* L.). Revue canadienne de phytotechnie 75:505-509.

Naraghi, L., Heydari, A., Rezaee, S., and Razavi, M. 2012. Biocontrol Agent *Talaromyces flavus* Stimulates the Growth of Cotton and Potato. Journal of Plant Growth Regulation 31:471–477

Naraghi, L., Heydari, A., Rezaee, S., Razavi, M., and Afshari-Azad, H. 2010. Biological control of Verticillium wilt of greenhouse cucumber by *Talaromyces flavus*. Phytopathologia Mediterranea 49:321−329.

Nicola, L., Tosi, S., and Savini, D. 2014. In vitro evaluation of nematophagous activity of fungal isolates. Journal of Basic Microbiology 54.

Nicoletti, R., and De Stefano, M. 2012. *Penicillium restrictum* as an Antagonist of Plant Pathogenic Fungi. Dynamic Biochemistry, Process Biotechnology and Molecular Biology 6:61-69.

Nihorimbere, V., Ongena, M., Smargiassi, M., and Thonart, P. 2011. Beneficial effect of the rhizosphere microbial community for plant growth and health Biotechnology, Agronomy and Society and Environment 15:327-337.

Nitzan, N., Cummings, T. F., and Johnson, D. A. 2008. Disease potential of soil- and tuber-borne inocula of *Colletotrichum coccodes* and black dot severity on potato. Plant Disease 92:1497-1502.

Nordskog, B., Nærstad, R., Herrero, M. L., Sundheim, L., and Hermansen, A. 2008. Fungal pathogens in Norwegian lettuce fields. Acta Agriculturae Scandinavica, Section B - Plant Soil Science 58:230-235.

Oyarzun, P. J., Dijst, G., Zoon, F. C., and Maas, P. W. T. 1997. Comparison of soil receptivity to *Thielaviopsis basicola*, *Aphanomyces euteiches*, and *Fusarium solani* f. sp. *pisi* causing root rot in pea. Phytopathology 87:534-541.

Papasotiriou, F. G., Varypatakis, K. G., Christofi, N., Tjamos, S. E., and Paplomatas, E. J. 2013. Olive mill wastes: A source of resistance for plants against *Verticillium dahliae* and a reservoir of biocontrol agents. Biological Control 67:51-60.

Park, J. O., Hargreaves, J. R., Cole, A. L. J., Ghisalberti, E. L., Gams, W., and Sivasithamparam, K. 2001. Cuticular disruption and mortality of *Caenorhabditis elegans* exposed to culture filtrate of *Byssochlamys nivea* Westling. Nematology 4:355–363.

Paulitz, T. C., and Baker, R. 1988. Interactions between *Pythium nunn* and *Pythium ultimum* on bean leaves. Canadian Journal of Microbiology 34:947-951.

Peethambaran, C. K., and Singh, R. S. 1977. Survival of different structures of *Pythium* spp. in soil. Indian Phytopathology 30:347-352.

Peng, Y.-H., Chou, Y.-J., Liu, Y.-C., Jen, J.-F., Chung, K.-R., and Huang, j. w. 2017. Inhibition of cucumber *Pythium* damping-off pathogen with zoosporicidal biosurfactants produced by *Bacillus mycoides*. Journal of Plant Diseases and Protection 124:481-491.

Petkowski, J. E., de Boer, R. F., Norng, S., Thomson, F., and Minchinton, E. J. 2013. *Pythium* species associated with root rot complex in winter-grown parsnip and parsley crops in south eastern Australia. Australasian Plant Pathology 42:403-411.

Plaats-Niterink, A. J. 1975. Species of *Pythium* in the Netherlands. Netherlands Journal of Plant Pathology 81:22-37.

Plaats-Niterink, A. J.1981. Monograph of the genus *Pythium*. Studies in Mycology 21:1-244.

Pryor, B. M., and Gilberston, R. L. 2000. Molecular phylogenetic relationships amongst *Alternaria* species and related fungi based upon analysis of nuclear ITS and mt SSU rDNA sequences. Mycological Research 104:1312-1321.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., and Glöckner, F. O. 2013. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. Nucleic Acids Research 41:D590-596.

Raaijmakers, J. M., Paulitz, T. C., Steinberg, C., Alabouvette, C., and Moënne-Loccoz, Y. 2008. The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. Plant and Soil 321:341-361.

RAP. 2017. Réseau d'avertissement Phytosanitaire (RAP), Bulletin d'information N.2, 25 juillet 2017.

Robideau, G. P., De Cock, A. W., Coffey, M. D., Voglmayr, H., Brouwer, H., Bala, K., Chitty, D. W., Desaulniers, N., Eggertson, Q. A., Gachon, C. M., Hu, C. H., Kupper, F. C., Rintoul, T. L., Sarhan, E., Verstappen, E. C., Zhang, Y., Bonants, P. J., Ristaino, J. B., and Levesque, C. A. 2011. DNA barcoding of oomycetes with cytochrome c oxidase subunit I and internal transcribed spacer. Molecular Ecology Resources 11:1002-1011.

Rodrigo, S., Santamaria, O., Halecker, S., Lledó, S., and Stadler, M. 2017. Antagonism between *Byssochlamys spectabilis* (anamorph *Paecilomyces variotii*) and plant pathogens: Involvement of the bioactive compounds produced by the endophyte. Annals of Applied Biology 171:464–476.

Rong, X., and McSpadden Gardener, B. 2013. Draft genome sequence of *Cryptococcus flavescens* strain OH182.9_3C, a biocontrol agent against fusarium head blight of wheat. Genome Announcements 1:e00762-00713.

SAS Institute Inc. 2017. JMP® 13 Predictive and Specialized Modeling, Second Edition. SAS Institute Inc., Cary, NC.

Sauvage, H., Moussart, A., Bois, F., Tivoli, B., Barray, S., and Laal, K. 2007. Development of a molecular method to detect and quantify *Aphanomyces euteiches* in soil. FEMS Microbiology Letters 273:64-69.

Schroeder, K. L., Okubara, P. A., Tambong, J., Lévesque, C. A., and Paulitz, T. C. 2006. Identification and quantification of pathogenic *Pythium* spp. from soils in eastern Washington using real-time polymerase chain reaction. Phytopathology 96:637-647.

Stanghellini, M. E., and Hancock, J. G. 1970. The Sporangium of *Pythium ultimum* as a Survival Structure in Soil. Phytopathology 61:157-164.

Stanghellini, M. E., and Burr, T. J. 1973a. Effect of soil water potential on disease incidence and oospore germination of *Pythium aphanidermatum*. Phytopathology 63:1496-1498.

Stanghellini, M. E., and Burr, T. J. 1973b. Germination in vivo of *Pythium aphanidermatum* oopores and sporangia. Phytopathology 63:1493-1496.

Sullivan, P. 2004. Sustainable management of soil-borne plant diseases: soil systems guide. http://www.attra.org/attra-pub/soilborne.html. [Accessed 2018].

The Weather Network. 2018. https://www.theweathernetwork.com/forecasts/statistics/precipitation/cl7026916/caqc2469. [Accessed 2018]

Thines, M., and Choi, Y. J. 2016. Evolution, diversity, and taxonomy of the Peronosporaceae, with focus on the genus *Peronospora*. Phytopathology 106:6-18.

Trivedi, P., Delgado-Baquerizo, M., Trivedi, C., Hamonts, K., Anderson, I. C., and Singh, B. K. 2017. Keystone microbial taxa regulate the invasion of a fungal pathogen in agro-ecosystems. Soil Biology and Biochemistry 111:10-14.

Van Bruggen, A. H. C., and Semenov, A. M. 1999. A new approach to the search for indicators of root disease suppression. Australasian Plant Pathology 28:4-10.

Van der Heyden, H., Wallon, T., Lévesque, C. A., and Carisse, O. In press. Detection and quantification of *Pythium tracheiphilum* in soil by multiplex real-time qPCR. Plant Disease In press.

Vero, S., Garmendia, G., Gonzalez, M. B., Bentancur, O., and Wisniewski, M. 2013. Evaluation of yeasts obtained from Antarctic soil samples as biocontrol agents for the management of postharvest diseases of apple (*Malus x domestica*). FEMS Yeast Research 13:189-199.

Viggiano, J. R., de Freitas, L. G., and Lopes, E. A. 2014. Use of Pochonia chlamydosporia to control Meloidogyne javanica in cucumber. Biological Control 69:72-77.

Vivoda, E., Davisr, M., Nunez, J. J., and Guerard, J. P. 1991. Factors affecting the development of cavity spot of carrot. Plant Disease 75:519-522.

Wakeham, A. J., and Pettitt, T. R. 2017. Diagnostic tests and their application in the management of soil- and water-borne oomycete pathogen species. Annals of Applied Biology 170:45-67.

Wallenhammar, A.-C., Almquist, C., Söderström, M., and Jonsson, A. 2012. In-field distribution of *Plasmodiophora brassicae* measured using quantitative real-time PCR. Plant Pathology 61:16-28.

Wang, P. H., and White, J. G. 1997. Molecular characterization of *Pythium* species based on RFLP analysis of the internal transcribed spacer region of ribosomal DNA. Physiological and Molecular Plant Pathology 51:129-143.

Wang, P. H., Wang, Y. T., and White, J. G. 2003. Species-specific PCR primers for *Pythium* developed form ribosomal ITS1 region. Letters in Applied Microbiology 37:127-132.

Waterhouse, G. M. 1967. Key to *Pythium* Pringsheim. Mycological Papers. 109:1-15.

Weller, D. M., Raaijmakers, J. M., Gardener, B. B., and Thomashow, L. S. 2002. Microbial populations responsible for specific soil suppressiveness to plant pathogens. Annual Review of Phytopathology 40:309-348.

White, T.J., Bruns, T.D., Lee, S.B. and Taylor, J.W. (1990) Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. Pages 315-322 in: PCR Protocols: A Guide to Methods and Applications. M. A, Innis, D. H., Gelfand, J. J., Sninsky and T. J., White, eds. Academic Press, New York.

Wiyono, S., and Agustina, E. R. 2013. Use of yeast antagonist *Cryptococcus terreus* to control petal blight of dendrobium caused by *Curvularia pallescens*. Journal of ISSAAS (International Society for Southeast Asian Agricultural Sciences) 19:41-48 ref.28.

Wu, Z., Hao, Z., Zeng, Y., Guo, L., Huang, L., and Chen, B. 2015. Molecular characterization of microbial communities in the rhizosphere soils and roots of diseased and healthy *Panax notoginseng*. Antonie Van Leeuwenhoek 108:1059-1074.

XiaoJia, H., MingHai, Z., ChangBing, H., LiHua, X., YuGui, C., YinShui, L., ShengYi, L., XiaoPing, F., ChunLei, Z., and Xing, L. 2010. Isolated fungal strains of inhibiting sclerotia germination and primarily study of biocontrol. Chinese Journal of Oil Crop Sciences 32:567-570 ref.514.

Zarandi, D. M., and Sharzei, A. 2015. First Report of Lemon Verbena Leaf Spot Caused by *Ulocladium chartarum* in Iran. Plant Disease (Disease Notes) 99:1186.

Zhao, S. S., Zhang, Y. Y., Yan, W., Cao, L. L., Xiao, Y., and Ye, Y. H. 2017. *Chaetomium globosum* CDW7, a potential biological control strain and its antifungal metabolites. FEMS Microbiology Letters 364:fnw287.

Zhou, T., and Paulitz, T. C. 1993. In vitro and in vivo effects of *Pseudomonas* spp. on *Pythium aphanidermatum*: zoospore behavior in exudates and on the rhizoplane of bacteria-treated cucumber roots. Phytopathology 83:872-876.

Zouari, I., Jlaiel, L., Tounsi, S., and Trigui, M. 2016. Biocontrol activity of the endophytic *Bacillus amyloliquefaciens* strain CEIZ-11 against *Pythium aphanidermatum* and purification of its bioactive compounds. Biological Control 100:54-62.