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A STUDY OF FUNGAL LEAF DECOMPOSITION IN RELATION TO BIOLOGICAL CONTROL OF THE APPLE SCAB PATHOGEN, *VENTURIA INAEQUALIS*.

by

Julie Bernier

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

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Short title

The biological control of apple scab and fungal leaf decomposition

Julie Bernier ©

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FOREWORD

This thesis is made up of four sections. The first section presents a general overview of the problem, the review on the previous work on this subject and the purpose of this study. Part two is on the collection of fungi from apple leaves and part three contains the different experiments done with the previous collection. The last part consists of a general discussion and conclusion.

The general thesis format has been approved by the Faculty of Graduate Studies and Research of McGill University and follows the conditions outlined in the "Guidelines Concerning Thesis Preparation", section B, "Manuscripts and authorship" which are as follows:

> The candidate has the option, subject to the approval of the Department, of including as part of the thesis the text, or duplicated published text (see below), of an original paper, or papers. Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (e.g. in appendices) to allow a clear and precise jugedment to be made of the importance and originality of the research reported. The thesis should be more than a mere collection of manuscripts published or to be published. It must include a general abstract, a full introduction and literature review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interest of cohesion.

Although all the work presented here was the responsibility of the candidate, the project was cosupervised by Dr. Odile Carisse, Agriculture and Agri-Food Canada (St-Jean-sur-Richelieu) and Dr. Timothy Paulitz, McGill University (Montréal). The manuscripts to be published from this work will be co-authored by O. Carisse and T. Paulitz.

ABSTRACT

STUDY ON FUNGAL LEAF DECOMPOSITION IN RELATION TO BIOLOGICAL CONTROL OF THE APPLE SCAB PATHOGEN, *VENTURIA INAEQUALIS.*

M.Sc. Julie Bernier Plant Science

Venturia inaequalis, the causal agent of apple scab, overwinters in apple leaves on the orchard floor. To develop a control strategy based on the prevention of the maturation of overwintering pseudothecia , a sampling of fungi colonizing dead apple leaves was conducted from different orchard floors in Quebec during the spring and fall of 1993. A total of 345 different isolates were obtained, from which fifteen genera have never been previously recorded as colonizers of apple leaves in North America. Small differences were detected in genera richness among orchards but the fungal composition of each orchard was fairly unique. Different tests on growth on amended media and leaf decomposition demonstrated that leaf degradation is not a reliable parameter alone to screen antagonist against *V. inaequalis*. No significant relation between growth on amended media, leaf rheology and ascospore inhibition was detected. However, 40 fungi reduced significantly ascospore production more than 87% compared to the control (*V. inaequalis* only). Of these antagonists, 30% decomposed apple leaves, suggesting that competition for the substrate is involved in the mode of action of at least one third of the antagonits detected. Other possible modes of antagonism are discussed.

RÉSUMÉ

UNE ÉTUDE DE LA DÉCOMPOSITION DES FEUILLES PAR DES CHAMPIGNONS SAPROPHYTES EN RELATION AVEC LE CONTRÔLE DU PATHOGÈNE DE LA TAVELURE, *VENTURIA INAEQUALIS.*

M.Sc. Julie Bernier Plant Science

Le champignon causant la tavelure du pommier, *Venturia inaequalis,* hiverne dans les feuilles de pommiers de la littière des vergers. Pour prévenir la formation et la maturation des pseudothèces, des échantillonnages de champignons colonisant ces feuilles ont été rammassées dans divers vergers du Québec au printemps et à l'automne de 1993. Un total de 345 isolates differents ont été isolés, dont quize genres n'ayant jamais été précédemment enregistrés comme coloniseurs de feuille de pommiers en Amérique du Nord. Peu de différences ont été détectés dans la richesse en genres des divers vergers mais la composition fongique de chaque verger s'est avérée unique. Différents tests sur la décomposition des feuilles ont démontré que ce paramètre n'est pas un bon indicateur pour le cribblage d'antagonistes contre *V. inaequalis.* Il n'y a pas eu de relation significative entre la croissance des isolats sur des milieux spécialement amendés, la rhéologie des feuilles et l'inhibition d'ascospores. Néanmoins, 40 champignons ont significativement réduit la production d'ascospores de plus de 87% comparativement au témoin (*V. inaequalis* seulement). De ces antagonistes, 30% ont décomposés les feuilles, suggérant que la compétition pour le substrat semble être impliquée dans le tiers de l'antagonisme détecté.

La mise en évidence de différents mode d'actions sont discutés.

ACKNOWLEDGEMENTS and DEDICATION

I want to thank the Pest Management Alternatives Office for their financial support.

Among all the people who helped me, I want first to tell all my gratefulness to Dr. Carisse for her support, the statistical analysis, the progress in my own scientific cheminement, and mainly for all the time she spent with me for unexpected problems. I also want to express all my recognition to Dr. Paulitz for the fungal identifications, his precious ideas and its expertise.

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For Guy, all the possible written words are not strong enough to express the feeling that I have when I think of all my difficulties he turned in a positive way to encourage me. Thank you ti-bâs.

To all graduated students who will refer to
this thesis to execute their own researches.

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1. GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

1.1.1 History of plant disease control

There has been concern about plant diseases since agriculture began. In early times, growers used cultural techniques to reduce disease incidence. They removed attacked plants and selected the seeds from healthy plants only. Today, because of industrialization, the improved living conditions and the increase in population density, the need to produce more and high quality agricultural products is necessary to meet the food and economic needs of the growers, the nations and the world (Agrios, 1976). Traditionally, the most effective and common way to fight against disease is to use chemical products such as fungicides (Boland, 1990). The use of fungicides has many positive effects; they are effective, easy to use, are generally not expensive and they are often broad spectrum. However, it appeared obvious in the 1960s that these products had several negative impacts. In fact, they persist in the food chain, are responsible for pollution, and can induce resistance within the pathogen population (Lewis and Papavizas, 1991). They also lost some of their previous advantages. Their utilization cost is increasing every year. The average of costs associated with discovery and registration of a pesticide was $2,494,000 in 1964 and reached $18,000,000 in 1976 (Fry, 1982). Some fungicides, like benomyl and dodine, lost their efficacy against apple scab because of resistance (Carisse and Pelletier, 1994; Jones, 1981). Finally, their utilization is not as easy as it was. With the increasing knowledge in epidemiology, complexity has increased with the use of different management techniques such

as tables for predicting infection. In 1975, it was estimated that agricultural operations accounted for 60% of the $36.3x10^4$-$45.4x10^4$ kg of pesticides applied annually in the U.S.A. On a per unit area basis, apples receive more pesticide than any other U.S. crop (Andrews and Kenerley, 1978) and apples are also the most world wide important crop for which fungicides are used intensively (Fry, 1982). Today, for economical, ecological or social reasons; the need to develop alternative ways to control plant pathogens is becoming a priority for scientific researchers all around the world (Boland,1990).

1.1.2 Alternatives to chemical control

There are relatively few alternatives to chemical control, namely, cultural, genetic and biological control.

One of these alternatives is the use of cultural practices. Some of these methods include elimination of the pathogen from the plant or from its surroundings (exclusion), other methods include creating conditions unfavorable to the pathogen or favorable to antagonists (such as manure applications, sanitation, improvement of growing conditions of plants and rotation) (Lewis and Papavizas, 1991).

Control by host resistance is an attractive approach for suppressing plant disease with less or without fungicides. Using resistance requires no action from the growers, it is not harmful to the environment and is also compatible with other management techniques. Resistance is the characteristic of a plant which suppresses the pathogen and its development. The magnitude of the effect can be high, moderate or low and the inheritance can be conditioned by one or several genes. Although this method contributes to disease

suppression, there are some problems affecting its usefulness. One of the limiting factors is the adaptation of the pathogens to overcome host resistance. Consequently, part of the host resistance is effective for relatively short time, and is not always stable, especially when resistance is monogenic. For example, the rust resistant wheat variety Eureka was released in 1938 and in 1941, the plant began to be rusted (Fry, 1982) because of pathogen adaptation.

Biological control in plant pathology can be broadly described using Garrett's definition "any condition under which or practice whereby, survival or activity of a pathogen is reduced through the agency of any other living organism (except man himself), with the result that there is a reduction in the incidence of the disease caused by the pathogen" (Fry, 1982). A more restrictive definition is the inoculum reduction of the pathogen by one or many microorganisms (Lewis and Papavizas, 1991). In some cases, antagonists to the pathogens are added in the system and in other cases, the environment is modified to favor antagonists. The pioneering studies on biocontrol were initiated as early as in 1920, where antagonistic fungi were introduced into forest nursery soils to reduce damping-off of pine seedlings (Boland, 1990). Biological control occurs naturally, but the knowledge about how it operates is usually insufficient to allow the exploitation of that phenomenon in agriculture. Natural biocontrol is probably operative and severe diseases develop primarily when natural control mechanisms are broken (Fry, 1982)

1.1.3 Biological control on the phyllosphere

Because of the unsuccessful and sporadic research during the 1930s to 1950s on the introduction of foreign antagonists and on the research of antibiotics producing microorganisms, plant pathologists and researchers were

discouraged and had negative attitudes towards biocontrol (Cook, 1981). It was only in 1963, at an international symposium on ecological soil-borne plant pathogens, in Berkeley, California that the research on biocontrol became an organized discipline (Cook, 1981).

Many studies and practices of biocontrol were directed toward diseases induced by soil-borne pathogen because of the stable environment of the soil, but during the last twenty years, an increased interest has been developed in relation to plant surfaces as habitat for microorganisms although their presence has been known for more than a century (Pugh and Buckley, 1971). A plant contains several ecological microhabitats that represent unique micro climatic and nutritional conditions. Terms such as rhizoplane and rhizosphere for the roots and phylloplane, phyllosphere for the leaves, are used to describe the uniqueness of these habitats and their influence on the growth and survival of pathogenic and saprophytic organisms (Boland, 1990). These organisms can be pathogenic or saprophytic and could be used as biocontrol agents. However biological control in phyllosphere is generally less successful than in the rhizosphere, despite the interesting results of Jarvis and Slingby (1977) on the control of powdery mildew of cucumber by *Ampelomyces quisqualis*. According to Cullen and Andrews (1984[a]), there are four reasons why practical biocontrol strategies applicable to the aerial plant pathogens have not been forthcoming. First, there are gaps in the basic knowledge of the organization and function of microbial communities of the leaves and their relationship to pathogenesis. The second reason is that there were few research programs in which biocontrol agents were identified, intensively tested and improved. The third point is that unfortunately, there is a tendency to adopt the same biocontrol strategies for the phyllosphere as those developed for the control of soilborne pathogens. Effectiveness of the antagonist is often affected by the chemical treatments

especially those which are known to have a wide spectrum of activity against pathogens. Finally, several studies have been done on the effect of fungicides on non-parasitic microflora. The consequences of fungicides on the interactions between saprophytes and pathogens is a shift in the natural balance of these organisms as well as senescence effects on the leaf decomposition (Dickinson, 1973). More specifically, Hislop and Cox (1969) found that captan, a commonly used fungicide, reduced the microflora on apple buds and apple leaves. Even if the sprays were stopped, the number and type of fungi returned to normal only after a few months. In 1978, Andrews and Kenerley used a pesticide program including applications of an insecticide, a bactericide and a fungicide to study its effect on non-target epiphytic microbial populations of living apple leaves. Populations of bacteria, yeast and fungi were reduced 10- to 1000-fold, and the fungal population was less diverse, resulting in a different composition. In 1979, they found that the same pesticide program also affected the microflora of apple leaf litter. The population reduction recorded in 1978 did not recover until snow cover had melted in March even if the treatments ended in September. So seasonal treatment can have a prolonged impact of 5 to 7 months on non-target micro-organisms. This long lasting effect is caused by the large disturbance of the flora instead of the long persistance of the pesticides. This suggests that if the phylloplane microflora is restricted by fungicides, then antagonism towards pathogens is also affected and it may be difficult to include biological control in a fungicide program.

The difference in the physiology and the morphology of a leaf compared to a root can also be a reason why practical biocontrol strategies applicable to the aerial plant pathogens have not been forthcoming. This can be explained by the complex topography of the leaf with its hills, craters, veins, compared to the

fairly smooth surface of the root (Andrews, 1992). There is the harsh micro climate of the leaf compared to the buffering properties of the soil, and the nutrients which originate from exogenous sources for the leaf compared to the root which is the primary nutrient reservoir (Andrews, 1992). Antagonistic microorganisms can come from the phylloplane of the infected leaf or from another habitat. In the latter situation, the establishment of the antagonist can be more risky because the environmental conditions near the leaves can vary drastically and because the antagonist has to be able to survive, to colonize the leaves and express its antagonistic properties. Kinkel et al. (1989), investigated the influences of introduced microbial populations on epiphytic leaf microflora. They concluded that the introduction of populations altered the fungal immigration in the field. However, there was no long term effects of introduced populations on fungal community size. They also showed that immigration is necessary but not sufficient for the establishment of a microorganism within the phylloplane community.

1.2.4 Antagonistic effects of biocontrol agents

The antagonistic effect can be classified in the following categories: competition, antibiosis and mycoparasitism or hyperparasitism (Cook, 1981).

Competition can be defined as the injurious affect of one organism on another because of the utilization or removal of some external resources from the environment (Baker, 1968). Competition may be for nutrients (nitrogen, carbon, vitamins, leachates from the host and microbes, pollen, and insect honeydew) and also for space even if most estimates of microbial coverage on leaf surface are well below 1% (Cullen and Andrews, 1984[a]). There is some indirect evidences which suggest that competition plays a role in natural biocontrol. In fact, some experiments showed the possible presence of competition when the

antagonistic effect of a biocontrol agent disappeared when using only the cell filtrates to inhibit the pathogen. For example, Boland and Hunter (1988) showed that nutrient competition may be implied in the disease control of white mold of bean caused by *Sclerotinia sclerotium*. It was found that *Alternaria alternata* and *Cladosporium cladosporioides* greatly restricted the disease incidence in inoculated plots, but they did not observe any inhibition of ascospore or mycelial growth when those fungi were put together on agar plates. This suggests that these two organisms did not exert their influence directly on *Sclerotinia* through parasitism or antibiosis.

Antibiosis can be explained by the production of secondary metabolites, such as antibiotics. This mode of action is mainly found by using co-culture tests or cell-free culture filtrates affect the germination, growth of pathogens, or disease incidence. A number of phylloplane microorganisms (fungi, yeast and bacteria) have been reported to produce antibiotics in vitro. Of the filamentous fungi inhabiting leaf surfaces, species of *Alternaria, Botrytis* , *Aureobasidium* and *Trichoderma* are antibiotics producers (Blakeman and Fokkema, 1982). However, there are some conditions necessary for antibiosis. Its production requires adequate nutrition for the antagonist, especially the appropriate carbon source. There is some evidence that antibiosis is effective in biological control. Some fungal studies gave positive results with the use of antibiosis: The inhibition of *Venturia inaequalis* by *Chaetomium globosum,* the zones of inhibition of *A. tumefaciens* in presence of *A. radiobacter* was a good indicator of field performance of *A. radiobacter* in controlling *A. tumefaciens* on peaches (Fravel, 1988). Despite these promising results, antibiosis in culture media does not necessarily mean that this phenomenon occurs in nature, and finally, the inactivation of the antibiotic by different factors may occur. The literature has

a few examples demonstrating the lack of correlation between antibiosis and biological control (Baker, 1968). For example, in an *in vitro* screening of 238 bacterial and yeast isolates, no correlation was found between inhibition *in vitro* and the biocontrol of *Bipolaris maydis* on maize leaves. Similar results were also obtained for the ability of hundreds of actinomycetes in controling *Ceratocystis ulmi in vitro* compared to the disease control on elm trees (Fravel, 1988).

Mycoparasitism or hyperparasitism are used to describe the interaction where a fungus is the host for another parasitic fungus. Hyperparasitism refers to the parasitism exerted by a fungus on other fungi which are themselves parasites of higher plants. Hyperparasites attack hyphae, sporulating and fruiting structures of the pathogen in the field, thus reducing risk of infection and plant pathogen inoculum. Specialized hyperparasites can affect plant pathogens in two ways: penetration of the fungal tissues and production of metabolic substances which result in the destruction and lysis of the hyphae or spores is one mechanism. The second method is the displacement of tissues of the pathogen within pustules or by the formation of crusts of mycelium which overlay fruiting structures. Less specialized hyperparasites restrict development of other fungi, including plant pathogens, by hyphal interactions involving coiling and penetration (Blakeman and Fokkema, 1982). Some important hyperparasitic fungi are *Darluca filum, Tuberculina vinosa,* and *Verticillium lecanii* on rusts, and *Ampelomyces quisqualis* to control cucumber powdery mildews (Blakeman, 1981).

Therefore, an important step in the utilization of an antagonist is its permanent establishment and colonization on the host plant. This is a prerequisite for efficacy independantly of the modes of action. Thus, the antagonist in question

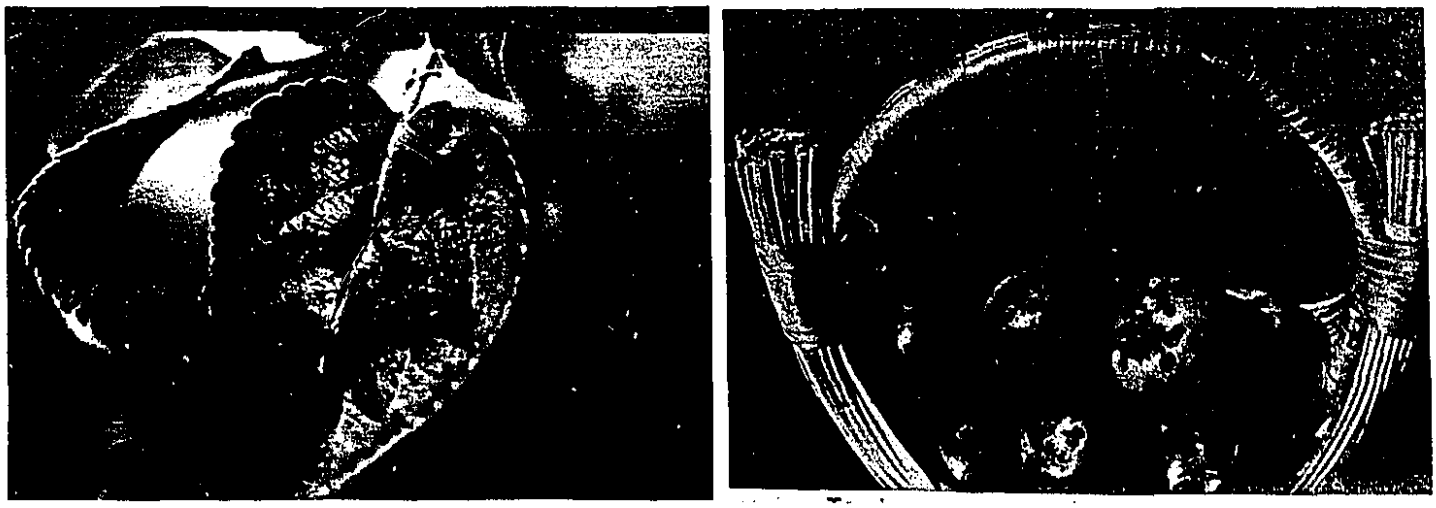
has to be well adapted to the environment to be effective (Bosshard *et al.*, 1987).

1.2 Review of literature

1.2.1 The apple scab disease

Of all the fungal diseases of pome and stone fruits, scab is the most important worldwide (Agrios, 1978). Fungi of the genus *Venturia* such as *V. inaequalis* on apples, *V. pirina* on pears, *V. cerasi* on cherries and *V. carpophila* on peaches have a particular economic importance, and the most important of these pathogens is *V. inaequalis* (Paul, 1981).

Venturia inaequalis (Cke) Wint. which causes apple scab is a Loculoascomycete in the order Pleosporales. This fungus is also named *Spilocaea pomi* because of its imperfect stage. This fungus affects principally the leaves, causing brownish-black spots. *Venturia* produces scab lesions, which appear as brownish-black, round spots. When the disease attacks the fruit, the skin of the apple can split within the area of the lesion and cracks can appear (Figure 1). These cracks may also provide an entrance for secondary pathogens like *Monilinia* sp. To conterreact increased loss of moisture at those sites, the apple can form corky layers but these do not keep pace with the growth of the fruit (Paul, 1981). The disease can also appear during storage.



a b

FIG 1: a-scab symptoms on apple leaves. b-Apples showing lesions caused by *Venturia inaequalis.*

Some studies indicate that *Venturia inaequalis* population is made up of several strains that differ to varying degrees in their physiologic characters. Roig (1987) described the five races named at present, a new sixth one being found in 1993 (Merwin et al. 1994). Some *Venturia* strains showed differences in their virulence among apple tree cultivars. Colony morphology, growth and dry weight of the fungus and the esterase isoenzymes can be used to distinguish the five races (Boone, 1971).

1.2.2 Life cycle and epidemiology of Venturia inaequalis

The apple scab pathogen, *Venturia inaequalis* (Cke) Wint. has two distinct phases in its life cycle. The first one, the saprophytic or perfect stage occurs when the fungus overwinters in infected apple leaf litter (Paul, 1981). From October, stroma composed of many hyphae deep in the palissade tissue can be observed. Then, the pseudothecia cavity is formed, and finally it erupts out of the epidermis. *Venturia inaequalis* is a heterothallic fungus. Isolates studies showed that there are two sterility groups or mating types (+ and -) both are

required to produce the sexual structures and spores. *Venturia* is also hermaphroditic, self-sterile, intra-group sterile and inter-group fertile (Keitt and Palmiter, 1938). The pseudothecia maturation occurs only in late fall and early spring; the mature pseudothecia bear asci containing 2-celled (the cells of unequal size) ascospores (sexual spores) of 11-15 x 5 µm in size (Viennot-Bourgin, 1949; Boone, 1971). James and Sutton (1982) observed that the development of asci and ascospores was initiated in the spring only after a dormant period of about 45 days. After this resting period, the pseudothecia mature rapidly according to two major factors which are not present or had no affect during the resting period. It was shown that a 100% relative humidity and temperature of 8°C to 12°C was optimum for pseudothecial development while 16°C to 18°C is optimum for ascospore maturation. According to Jeger and Butt (1983), pseudothecia matured most rapidly with high rainfall in November and high temperatures in spring, and were more abundant with low temperatures in November and low rainfall in spring. In spring, the fungus begins to forcibly eject its ascospores through the ostioles of the pseudothecia. Weather conditions such as rain and humidity, which favor the development of buds, also favor the ejection of ascospores, so there is a synchronization between plant phemology and apthogen life cycle (Alexopoulos, 1952). In North America, ascospore ejections seems to start after a rain of 3 to 15 hours in duration around mid May, and it stops in July (Viennot-Bourgin, 1949). With the help of the wind, the spores are dispersed in the air and land on new expanding green leaves which are more susceptible than older leaves.

V. inaequalis begins its parasitic or imperfect stage following the germination of the ascospores which cause the primary infection and the first scab lesions. The evolution of the disease occurs by the rapid formation of asexual spores (conidia) of 12-22 x 6-9 µm in size from the conidiophores, which cause the

secondary infections (Boone, 1971). These conidia germinate and give rise to hyphae that continue to grow and colonize the host over a wide range of temperature, with a minimum of 0°C to a maximum of 30°C; the incubation period being three weeks at 5°C and eight days at 19°C (Paul, 1981). The fungus propagates itself asexually throughout the spring and summer, producing several conidial generations (Alexopoulos,1952).

1.2.3 Control by fungicides

Over the last 50 years, apple scab has been controlled by fungicides. To produce commercially acceptable apples, eight to twenty fungicides applications are needed each growing season (Jones, 1985), representing approximatively 80 kg/ha annually of different chemical treatments (Merwin et al. 1994). In the 1970s, there were at least 17 different fungicides and some 30 brand-name formulations available to control apple scab (Merwin et al. 1994). It was also demonstrated by Andrews and Kenerley (1979) that fungicides also affect the composition of the non-target microorganisms, those organisms being implicated in natural biological control of scab. Since the extensive epidemiological studies of Keitt and his co-workers in the 1920's, only the development of Mill's infection periods in the 1940's has provided a workable knowledge for improving the efficiency in the scheduling fungicides applications (MacHardy and Jeger, 1983). Besides the problem of spraying management, we also have the problem of fungicide resistance. Since 1969, a year that corresponded to the first failure of dodine sprays against apple scab, other new fungicides have lost their effectiveness (Jones, 1981). It was demonstrated that tolerance to dodine and benomyl is associated with the continual and almost exclusive use of these fungicides for several years; 10 years for dodine and 3 years for benomyl (Jones, 1981). Today, the resistance phenomenon has been

recorded in many regions such as Canada, Michigan, Minnesota, New York and Virginia (Carisse and Pelletier, 1994; Jones, 1981). It is necessary to add that benomyl-tolerant strains of *V. inaequalis* are tolerant to thiophanate-methyl and are probably cross-tolerant to the other benzimidazoles (Jones and Walker, 1976).

In the begining of the 1980's, a large number of fungicides that inhibit ergosterol biosynthesis were tested in the United States and their use contributes to increase the selection pressure on pathogens like *V. inaequalis* (Jones, 1981). These fungicides include fenarimol, triforine, bitertanol, and prochloraz. More recently, Biggs and Warner (1990) found other promising sterol inhibiting fungicides, namely bitertanol, flusilazole and triflumizole. The pseudothecia of *V. inaequalis* are important as a source of new mutants because of possible genetic recombination among the two compatible strains needed for the sexual stage. Much attention has been paid to the alternation of fungicides to reduce resistance because the pathogen may develop resistance to both products (Jones, 1981).

1.2.4 Cultural control

Besides control by fungicides, there is another approach that was considered for apple scab: cultural control. One important aspect in the struggle against apple scab is to control the primary infection; which will prevent secondary infections. It was demonstrated that there is a relationship between the potential ascospore dose present in the orchard and the severity of scab during the season (MacHardy,1990). A productivity test done by Hirst and Stedman (1962) confirmed that leaf infection would be a better epidemiological parameter for scab severity than the fruit infection.

Because the pathogen overwinters in the fallen leaves, cultural control can be achieved through the management of fallen leaves, in autumn. Hirst and Stedman (1962), suggested that the ascospore dose could be determinated by the weight of leaf remaining in the spring and ascospore productivity. It was demonstrated that certain sanitation practices (leaf shredding, nitrogen enrichment) can reduce the potential ascospore dose (MacHardy, 1990). According to Miller and Rich (1968), nitrogenous fertilizers, lime and potassium phosphate applied in autumn hasten the decomposition of the leaf litter during the winter. This decomposition resulted in a reduction of the ascospore production of *V. inaequalis* and may reduce the severity of infection in the spring. The use of urea to control the perfect stage of *V. inaequalis* is also a way of control. Ross and Burchill (1968) found that urea treatment caused a reduction of scab infections by the high nitrogen content within the leaf, the toxicity of urea and the increase of antagonistic bacteria. This method may also reduce the overwintering inoculum from 70 to 99% through the suppression of pseudothecial development and enhancement of leaf decomposition (MacHardy and Jeger, 1983). Despite good results obtained with urea treatments, researchers continued to look for an alternative method to urea which has some negative environmental and cultural impacts, such as reducing winter hardening.

Another management practice could be to favor the fast decomposition of fallen leaves by encouraging the microbial litter flora. Heye and Andrews (1983) observed that the leaves inoculated with *Athelia bombacina*, a fungal leaf inhabitant, became softer and may have decomposed faster, supporting less development of pseudothecia, but they did not research this further.

1.2.5 Control by host resistance

The control of disease by host resistance has also been an approach since the end of the 19th century, when the first scab resistant varieties were developed. The researchers believed that cross-breeding presented the most likely solution for developing scab resistant varieties. In 1945, a cooperative program of scab resistance breeding was developed at the University of Illinois and in 1955, a program began to determine the relationship amongst scab resistance genes. One of the findings was that susceptibility or resistance is determined by the contributions of both host and pathogen (Williams and Kuc, 1969). It was also found that for each gene in the plant that conditioned resistance, there was a specific and related gene for virulence in the parasite. It was also suggested that only mutations to resistance in the host and mutations to virulence in the parasite would have possible value in the evolution of host-parasite system. A similar gene-for-gene relationship had been found in the apple variety McIntosh and *V. inaequalis,* which indicates that the host resistance is conditioned by a single gene. For each lesion-conditioning gene in the pathogen, there was a corresponding gene for the susceptibility or resistance in the host (Bagga and Boone, 1968). By now, there are at least two named apple scab resistant cultivars selected from St-Jean-sur-Richelieu: Richelieu and Rouville (Granger, 1991) and four from Cornell/Geneva/ and Purdue/Rutgers/Illinois breeding program: Goldrush, Enterprise, Freedom and Liberty (Merwin et al. 1994). Because *V. inaequalis* population is an important source of mutation, this approach, involving monogenic resistance should be considered carefully, since a new cultivar may be resistant only for a few years because of the possible pathogen mutations. Therefore, of the six pathogenic strains of *V. inaequalis*, five of them can overcome certain genes for resistance. In fact, of the fifty apple scab resistant cultivars, 39 contain the V_f single gene

(Merwin et al. 1994). This finding emphasized the need to diversify sources of resistance. This is why another approach would be to work with a lower level of resistance conditioned by many genes (polygenic horizontal resistance). This type of resistance shows some symptoms of different levels of disease on the host, but should be more stable overtime (Fry, 1982).

Disease management can be effective with integrated approaches, horizontal resistance being one of the elements that should reduce the need for fungicides applications. Another question is whether scab resistant cultivars are a viable alternative for commercial apple production when the taste of consummers changes every few years.

1.2.6 Biological control

Studies were conducted on alternative ways to control *Venturia inaequalis.* Many different studies were conducted on biocontrol of apple scab, the pioneer being Cinq-Mars in 1949. Ross (1953) continued with the ideas of Cinq-Mars. Ross worked with many parameters starting with the establishment of a collection of apple leaf microflora. He tested these isolates for antibiotic production and decomposition of apple leaves. Only *Penicillium* sp. was found to have antibiotic activity. This characteristic was verified by Simard et al, (1957) who found that of the 34 isolates isolated from apple leaves, 12 isolates, most of them being the genus *Penicillium*, produced a zone in which the growth of the pathogen was inhibited. For the decomposition of apple leaf, only one unidentified fungus decomposed the substrate of apple scab fungus (Ross, 1953). No further work was done with these two collections of organisms.

Heye (1982) found a basidiomycete, *Athelia bombacina* and an ascomycete, *Chaetomium globosum,* also from the apple leaf microflora. These two fungi

prevented and inhibited pseudothecial formation or ascospores production in an *in vitro* and *in vivo* assay; *A. bombacina* being more effective than *C. globosum.*

Andrews and co-workers (1983), tested *Chaetomium globosum* and seven other microorganisms on the imperfect stage of *Venturia inaequalis.* They made a battery of tests for the detection of antagonism (*in vitro* and *in vivo* assays), and ranked and determinated the presumptive mode of action (hyperparasitism, volatile and nonvolatile antibiosis, and competition). In the *in vitro* assay, *C. globosum* was the only antagonist that reduced germination and germ tube elongation of *V. inaequalis.* In the *in vivo* assay, *C. globosum* was the most effective antagonist for lesion development, disease severity, and conidial production. Based on these experiments, they suggested the involvement of hyperparasitism by *C. globosum* . Further research demonstrated that the mode of action of *C. globosum* was antibiosis, the antibiotic chaetomin being identified (Cullen and Andrews, 1984b). Assays in orchards showed that *C. globosum* ascospore suspensions reduced scab infection by only 20% when applied every two weeks. Its poor capacity of colonization and the photodegradation of the product needed for antibiosis were suggested as reasons for the failure (Cullen *et al.*, 1984).

Another study was conducted by Heye and Andrews (1983) on the antagonism of *A. bombacina* and *C. globosum* against the perfect stage of *V. inaequalis.* The inhibition of ascospore production on incubated leaf discs was between 40 and 100%. For the outside incubations, no ascospores were produced on the leaves treated with *Athelia bombacina,* and the production decreased about 30% with *C. globosum. C. globosum* tested against the perfect stage of *V. inaequalis* was not as effective as *A. bombacina* . The leaves

treated with *A. bombacina* were about 60% softer and weighed about 50% less than the control (not treated leaves). It was also suggested that *A. bombacina* acts through nutrient competition and antibiosis. In 1990, Young and Andrews used an immunochemical stain to detect *Athelia bombacina* in apple leaf litter. They observed that even if the number of pseudothecia was not reduced by the presence of *Athelia*, the pseudothecia of *Venturia* did not mature and did not produce any ascospores. There was no evidence of direct parasitism, but it seems that *A. bombacina* produces cellulases and pectinases, which accounted for the acceleration of leaf decomposition. They suggested that the pseudothecial development may have been inhibited by the disruption of the leaf structure by *A. bombacina* (Young and Andrews, 1990).

On the other hand, *C. globosum* was found to be a poor epiphytic colonizer, and the chaetomin must be present in high concentration. The antibiotic was affected in the field by a wide range of physical and biotic factors (Boudreau and Andrews, 1987). Field study conducted in Quebec indicated that *A. bombacina* do not survive under our climatic conditions (Carisse, personal communication). Despite the promising results obtained by Andrews and others (Heye and Andrews,1983; Cullen and Andrews, 1984b; Young and Andrews, 1990) research on this biological control agent was not continued.

1.3 Objectives

Because of the intensive use of pesticides and because cultural control and host resistance alone are not commercially effective enough, serious thought is needed about incorporating biological control into apple scab management programs. Despite various reseach on biological control of apple scab, no biocontrol agent is available. Most of the screening programs used a fairly small collection of organisms and no potential agents suitable at least for the province of Quebec have been found since the first studies in the 1950's. For this reason, we made a large apple leaf microflora collection from Quebec orchards to increase the probability to find appropriate antagonists. We hypothesized that it is possible to reduce the ascospore production of *Venturia inaequalis* by the use of organisms found in the same niche as the pathogen, the apple leaf microflora.

To obtain an effective mean of control of apple scab, we thought that it was better to work with the perfect stage of the pathogen instead of the conidial state. It would be more difficult to control the asexual (conidial) stage because of the exponential nature of spore production. The saprophytic stage may be the weaker stage of the pathogen because this stage is a survival one.

Different approaches to biocontrol should also be considered. One approach is to hasten the microbial decomposition of leaves and destroy the leaf substrate in which pseudothecia are formed. To control apple scab, we searched for isolates from the fallen leaves capable of growing at low temperatures that may be adapted to leaf litter conditions in Quebec during the time pseudothecia are formed. We are focusing on this approach since the destruction of the primary inoculum is less difficult than controlling the pathogen during its phase of rapid

conidial formation. Studying the ecological community of the phyllosphere is also one of the principles cited by Andrews (1990) to consolidate research efforts on biological control. In this perspective, to improve biological control, we must first learn about species composition and its structure.

Furthermore, working with fungi from apple leaf litter permits us to find not only microorganisms from the living leaves but also fungi originating from the soil surface and thus, probable decomposers.

Working with leaf litter inhabitants has an advantage because of environmental conditions. On the soil surface, the leaves and their microflora are less subject to temperature variations, wind and solar radiation. These variations and the seasonal changes found on living apple leaf microflora (Pennycook and Newhook, 1981) may also be an important factor for the establishment and the capacity of the biocontrol agent to occupy the leaf over a prolonged period of time. It was also shown that some antimicrobial compounds in the host such as phenol may be an important selective component of the phylloplane environment. Conceivably, the changes in the secretion of those compounds as well as the concentrations of nutrient exudates may play an important role in the presence or absence of some microorganisms on the living leaves (Cullen and Andrews, 1984a). Applying a biocontrol agent on trees is more difficult and probably less successful than spraying the orchard floor because of the environmental aspects cited above. Finally, because microbial populations on leaves are affected, reduced and modified by fungicides, it is probably not worth trying to establish an antagonist during the growing season.

Even if the antagonistic effect of a biocontrol agent using the competition seems to play a relatively minor role in the organization of natural phylloplane communities, we thought that because of the life cycle of *Venturia inaequalis* this mode of action has an interesting perspective. In fact, since infection by conidia occurs in a short period of time (between 12 and 24 hours), the period of exposure of the pathogen to an antagonist for the process of competiton is very brief. In this case, unless the epiphytic antagonist is present in very high density, it can't respond before the pathogen penetrates the host and escapes (Cullen and Andrews, 1984[a]). We thought it would be a better to work with the saprophytic stage of the pathogen since the antagonist remains in contact with *Venturia* for a much longer period lasting from autumn to spring.

Using this approach, we hope to reduce the initial inoculum present in the spring and to delay the need for protective fungicide sprays. This aspect of the biological control being just one part of an integrated approach to apple scab management.

To accomplish this, our specific objectives are:

1. Isolation of fungi inhabiting senescent apple leaves on the orchard floor in early spring and late fall. These isolates will be used for other evaluations.

2. Evaluation of the isolates for their degradative enzymes activity and for apple leaf decomposition in relation to ascospores production *in vitro.*

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2. A SURVEY OF FUNGAL COMMUNITIES ISOLATED FROM DEAD APPLE LEAVES

2.1 Abstract

Venturia inaequalis, the causal agent of apple scab, overwinters in apple leaves on the orchard floor. Pseudothecia are formed in the leaves, and release ascospores in the spring. To find fungi that could colonize apple leaves and prevent the maturation of pseudothecia over the winter under Quebec conditions, we conducted an extensive sampling of fungi from six orchards during two seasons (spring and fall) of 1993. A total of 189 different isolates were obtained from the leaves collected in the spring and 156 from those collected in fall. From these isolates, forty-nine genera were identified, twenty-three genera were found only in the spring and eleven genera appeared only in fall. Most of the genera (75%) belonged to the Deuteromycetes. Fifteen genera have never been previously recorded as colonizers of apple leaves in North America. Only small differences were detected in genera richness among orchards. However, cluster analysis of genera demonstrated that the fungal composition of each orchard was fairly unique. Most genera were isolated equally over both seasons.

Key Words: apple leaves, biological control, fungal community, fungal ecology.

2.2 Introduction

In Quebec, apple scab caused by the fungus *Venturia inaequalis.* (Cke.) Wint., is the most important disease in apple production and effective control requires from 6 to 16 fungicide applications in one season (Jones and Aldwinckle, 1990). Besides the environmental problems of fungicide applications, the pathogen is becoming increasingly resistant to fungicides, particulary to benomyl, dodine and fenarimol (Jones, 1981; Carisse and Pelletier, 1994). Therefore, there is an increasing interest in alternative ways to control disease including biological control.

Because *V.inaequalis* grows as a saprophyte when it overwinters in the apple leaf litter, reseachers have looked for possible organisms colonizing dead apple leaves. Cinq-Mars (1949) and Ross (1953) collected apple leaf microflora to find potential natural antagonists of *V. inaequalis* . This approach was also pursued by Simard et al (1957), Andrews and Kenerley (1979), Heye (1982) and Andrews et al. (1983). From all these studies, one interesting potential antagonist, *Athelia bombacina* (Heye, 1982), was found in Wisconsin but has never been commercialized. Since the work of Andrews and Heye, this strategy has not been pursued. In addition, no work has focused on organisms adapted to the cold conditions of eastern Canada. Rather than testing biocontrol agents developed in warmer climates, we proposed to search for indigenous organisms in Quebec.

We hypothesized that potential antagonists are present on apple leaves and that sampling of several orchards should provide a large diversity of genera and increase the chance of finding potential biocontrol agents. Knowledge of the

diversity of apple leaf microflora is essential in developing a biological control agent against apple scab, particularly for orchard testing and for studies on fitness and adaptability of the biocontrol agent.

The objectives of this research were to study the fungal communities of dead apple leaves in a cool temperate climate (Quebec), to acquire knowledge of the diversity of the microflora and to make a collection of fungi to be subsequently tested for their potential as biocontrol agents.

2.3 Materials and methods

2.3.1 Sampling area

The sampling was done in six apple (*Malus pumila* Mill var McIntosh) orchards representing different apple growing regions in the province of Quebec, Canada (Figure 2). The orchards were situated at Covey-Hill, Deschambault, Freligshburg, Ile d'Orléans, St-Hilaire and St-Joseph. These orchards were abandoned for many years so no fungicides treatment could have affected the natural fungal microflora. Dead apple leaves lying on the ground were randomly collected twice in 1993. The first collection was done in the spring after snow melt between April 20 and April 23. The second collection was carried out just before the first snow fall in the fall, between November 9 and November 15. The leaves were stored in paper bags and refrigerated at 4°C until processed.

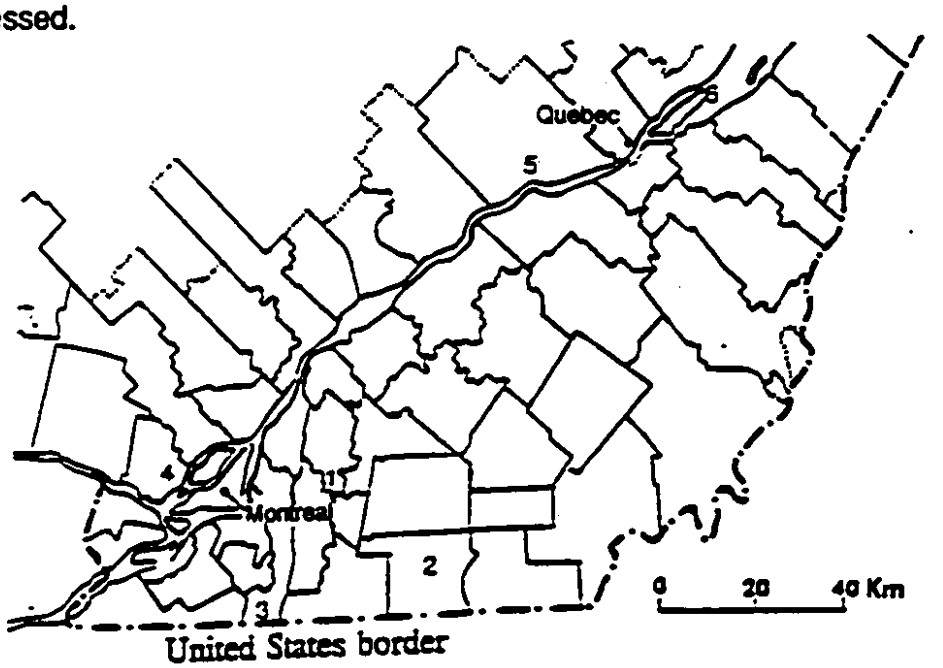

FIG 2. Location of the six orchards in Quebec from which dead apple leaves were collected. Orchard 1 was located in St-Hilaire, 2 in Frelighsburg, 3 in Covey-Hill, 4 in St-Joseph-du-Lac, 5 in Deschambault and 6 on Ile d'Orléans.

2.3.2 Isolation techniques

Two isolation methods were employed to collect the largest number of organisms. In the first method, for each orchard, randomly chosen leaves were placed in glass petri plates of 9 cm diameter containing a sheet of Whatman filter paper saturated with distilled water to provide a moist environment. The petri plates were incubated at each of eight different temperatures (-2, 0, 2, 4, 6, 10, 18, 24°C) for two to three weeks, four plates per temperature. The leaves were observed under a dissecting microscope and each mass of spores, fruiting bodies or mycelia was picked up and placed on half-strength V8 agar media (Dhingra and Sinclair, 1985) amended with 100 µg/ml of chlorotetracycline and 200 µg/ml of streptomycin. Using this procedure, the leaves were subjected to a large range of temperatures to favor the mesophilic as well as the psychrophilic fungi that could be active under low temperatures in the winter.

For the second method, 10 g of non-rinsed leaves were homogenized in a blender with 100 ml of distilled water. The homogenates were diluted from 10^{-1} to 10^{-3} with four replicates per dilution (Figure 3). Aliquots of 0.5 ml of these suspensions were spread on V8 agar (half strength, with antibiotics as described above) and on potato agar made from an infusion of 200 g of unpeeled potatoes boiled for 0.5 hour. The latter medium (used to favor the basidiomycetes) was also amended with 15 µg/ml of benomyl (Benlate 50 wp, diluted in 95% alcohol). Antibiotics and fungicide were added after autoclaving. In this method, there were four replicate plates for each medium for each dilution. There were 144 plates (6 orchards x 2 media x 3 dilutions x 4 replicates) incubated at each of the eight temperatures from -2 to 24°C for three to four weeks. Colonies with different morphologies were transferred to V8 medium.

Isolated fungi were identified according to their spore morphology (Barnett and Hunter, 1987).

FIG. 3: Different colonies of fungi resulting from the dilution plating of leaf decoction.

2.3.3 Data analysis

To examine the diversity of genera, the Margalef's richness index (RI) was computed for orchards and seasons (Ludwig and Reynolds,1988). Cluster analysis was used to compare the genera composition of the orchards, using binomial data (presence or abscence of the genera) with UPGMA-clustering computed with the software of Molecular Evolutionary Genetics Analysis (Romesburg, 1984; Sudhir et al, 1993). The frequency of distribution of the different genera for the different seasons was calculated. Fungi isolated with a frequency less than 2% were not included in this assay. For all analyses, only the identified fungi were used.

2.4 Results and discussion

From the spring collection, 189 different isolates were obtained and 71% of them were identified, giving 38 different genera (Table I). The most common genera were: *Alternaria, Cladosporium, Coniothyrium, Penicillium, Phoma, Trichoderma* and the order Mucorales. When the two methods were compared, *Aspergillus, Brachysporium, Curvularia, Geotrichum, Monilia, Mycogone, Tubercularia,* and yeasts were only isolated from homogenized leaves. The genera *Arthrobotrys, Cephalosporium, Chaetomium, Chaetophoma, Chalara, Diplodia, Hendersonia, Humicola, Hyalodendron, Paecelomyces, Pestalotia, Pyrenochaetae Rhinotrichum, Rhizoctonia, Selenophoma, Sphaeropsis, Varicosporium* and *Verticillium* were isolated only with the direct method. From the autumn collection 156 different isolates were obtained, and 69% were identified, giving rise to 26 genera (Table I). *Alternaria, Candida, Cladosporium, Coniothyrium, Epicoccum, Trichoderma,* the order Mucorales and yeasts were the most common. *Bactrodesmium, Ceratosporella, Chaetomium, Cylindrocarpon, Monilia, Papularia, Phoma and* the order Mucorales were only isolated from diluted leaf homogenates. *Melanconium, Sclerotinia, Trichoderma* and *Trichothecium* were isolated only with the direct method.

TABLE I: NUMBER OF FUNGAL ISOLATES GENERA RECOVERED FROM DEAD APPLE LEAVES IN QUÉBEC ORCHARDS IN SPRING AND FALL, 1993.

	SPRING			FALL			TOTAL OF METHODS		
GENERA	direct meth.	diluted meth.	total (%)[a]	direct meth.	diluted meth.	total (%)[a]	diluted meth.	diluted meth.	total (%)[a]
Alternaria	19	2	10.9	6	7	7.3	25	9	18.2
Arthrobotrys	1	-	0.5	-	-	-	1	-	0.5
Aspergillus	-	1	0.5	-	-	-	-	1	0.5
Aureobasidium	3	1	2.1	2	4	3.39	5	5	5.49
Bactrodesmium	-	-	-	-	2	1.12	-	2	1.12
Botrytis	2	1	1.6	-	-	-	2	1	1.6
Brachysporium	-	1	0.5	-	-	-	-	1	0.5
Candida	5	2	3.6	1	10	6.18	6	12	9.78
Cephalosporium	3	-	1.6	-	-	-	3	-	1.6
Ceratosporella	-	-	-	-	6	3.39	-	6	3.39
Chaetomium	1	-	0.5	-	1	0.56	1	1	1.06
Chaetophoma	1	-	0.5	-	-	-	1	-	0.5
Chalara	1	-	0.5	-	-	-	1	-	0.5
Cladosporium	21	9	15.6	12	30	23.7	33	39	39.3
Coniothyrium	12	2	7.3	1	14	8.47	13	16	15.7
Curvularia	-	1	0.5	-	-	-	-	1	0.5
Cylindrocarpon	-	-	-	-	1	0.56	-	1	0.56
Diplodia	1	-	0.5	-	-	-	1	-	0.5
Epicoccum	1	2	1.6	7	15	12.4	8	17	14.0
Fusarium	6	5	5.7	4	4	4.52	10	9	10.2
Geotrichum	-	2	2.1	-	-	-	-	2	2.1
Gilmaniella	-	-	-	-	1	0.56	-	1	0.56
Gliomastix	-	-	-	-	1	0.56	-	1	0.56
Hendersonia	1	-	0.5	-	-	-	1	-	0.5
Humicola	1	-	0.5	-	-	-	1	-	0.5
Hyalodendron	1	-	0.5	-	-	-	1	-	0.5
Melanconium	-	-	-	1	-	0.56	1	-	0.56
Monilia	-	1	0.5	-	1	0.56	-	2	1.06
Mortierella	-	-	-	2	1	1.69	2	1	1.69
order Mucorales	1	12	6.8	-	11	6.21	1	23	13.0
Mycogone	-	3	1.6	-	-	-	-	3	1.6
Paecilomyces	2	-	1.0	-	-	-	2	-	1.0
Papularia	-	-	-	-	2	1.12	-	2	1.12
Phoma	10	1	5.7	-	8	4.52	10	9	10.2
Penicillium	14	3	8.8	1	8	5.06	15	11	13.8
Pestalotia	1	-	0.5	-	-	-	1	-	0.5
Pyrenochaetae	2	-	1.0	-	-	-	2	-	1.0
Rhinotrichum	1	-	0.5	-	-	-	1	-	0.5
Rhizoctonia	1	-	0.5	1	1	1.12	2	1	1.62
Sclerotinia	-	-	-	1	-	0.56	1	-	0.56
Selenophoma	1	-	0.5	-	-	-	1	-	0.5
Sphaeropsis	1	-	0.5	-	-	-	1	-	0.5
Trichoderma	11	9	10.4	10	-	5.52	21	9	15.9
Trichothecium	-	-	-	1	-	0.56	1	-	0.56
Tubercularia	-	1	0.5	-	-	-	-	1	0.5
Ulocladium	-	-	-	1	1	1.12	1	1	1.12
Varicosporium	1	-	0.5	-	-	-	1	-	0.5
Verticillium	1	-	0.5	-	-	-	1	-	0.5
yeasts	-	5	2.6	1	14	9.04	1	20	11.6

[a] The total percentages were calculated from the total number of isolates of a given genus divided by the total number of isolates found in each season or for both seasons; multiplied by one-hundred. Results from the six orchards were pooled together.

For both seasons, of the genera isolated only once, fifteen were isolated with the direct plating method, and five with the dilution plating method. When the two collections were compared (Table I), 23 genera were found only in the spring, but 11 genera appreared only in the autumn collection.

Because the leaves were not surface sterilized for the dilution plating method, some of the organisms recovered may have come from the phylloplane, and may not be endophytes. Genera found only with the dilution plating method may not be common on or in the leaves, so they would be difficult to isolate from intact leaves because of competition from faster growing fungi. In fact, several fungi were isolated only once. On the other hand, it was demonstrated by Petrini (1991) that some epiphytes may, under appropriate conditions, colonize the interior of the host tissues. As an example, *Alternaria alternata* (Fr.) Keissler, and *Cladosporium cladosporioides* (Fr.) De Vries were recorded as phylloplane colonists that are able to penetrate the living plant tissues at the onset of the senescence process (Petrini, 1991). Therefore, because we collected dead leaves, it was assumed that there was no host specificity required for the endophytes to colonize the tissue due to the absence of defences present in living plants. Some epiphytic fungi may switch to a endophytic life style, first to decompose the leaf, and secondly to protect themselves against adverse conditions (snow cover) and also to reduce antagonistic activities by other competitive microorganisms.

According to Bessey (1950) and Barnett and Hunter (1987), out of the 49 genera obtained, 18 are recorded as saprophytes, 25 as both saprophytes and parasites, and 6 as parasites only. More specifically, according to Names of Plant Diseases in Canada (1992), of the 49 genera found, 11 are recorded as apple tree pathogens including, *Alternaria, Cladosporium, Coniothyrium,* and

Fusarium. However, we do not know whether these isolates are pathogenic species and some of these genera include up to hundreds of species.

In both methods (direct plating and dilution plating), different temperatures were used to recover the isolates. The fungi were classified into four groups, depending on the range of temperatures at which they were isolated (Table II).

TABLE II: GENERA OF FUNGI ISOLATED FROM DEAD APPLE LEAVES INCUBATED AT DIFFERENT TEMPERATURES

Cold Temperatures only (-2°C to 10°C)	Warm Temperatures only (18° and 24°C)	Warm and Cold Temperatures (-2 to 10; 18, 24°C)	Not influenced by Temperatures (-2°C to24°C)
Aspergillus	*Arthrobotrys*	*Aureobasidium*	*Alternaria*
Cylindrocarpon	*Brachysporium*	*Bactrodesmium*	*Candida*
Diplodia	*Chaetophoma*	*Botrytis*	*Cladosporium*
Geotrichum	*Chalara*	*Cephalosporium*	*Coniothyrium*
Hendersonia	*Curvularia*	*Ceratosporella*	*Epicoccum*
Selenophoma	*Gilmaniella*	*Chaetomium*	*Fusarium*
Sphaeropsis	*Gliomastix*	*Mortierella*	order mucorales
Tubercularia	*Humicola*	*Paecelomyces*	*Penicillium*
Varicosporium	*Hyalodendron*	*Papularia*	*Phoma*
Verticillium	*Melanconium*	*Pyrenochaeta*	yeasts
	Monilia	*Rhizoctonia*	
	Mycogone	*Ulocladium*	
	Pestalotia	*Trichoderma*	
	Rhinotrichum		
	Sclerotinia		
	Trichothecium		

The fungi only isolated at cold temperatures were present in very low frequencies, so we cannot say for certain that they would not be present at higher temperatures, if more samples were taken. The most common genera of fungi were isolated equally over all temperatures. Further testing would be

needed to see if we have isolated true psychrophiles. Nevertheless, using a range of isolation temperatures has increased the diversity of isolates.

The largest number of genera were found in St-Hilaire and St-Joseph in the spring collection (Figure 4). These two orchards also had the largest number of unique genera. However this trend was not seen in the fall collection.

To study the diversity of fungal genera for the different orchards, the genus richness, defined as the number of genera in a community, was calculated based on the Margalef richness index (formula: R=(G-1)/ln(n), where G is the number of genera and n is the total number of isolates found (Ludwig, 1988). Using the Margalef's index, the richness of each orchard over the two seasons combinated were ordered as follows: Ile d'Orléans, Frelighsburg, Covey-Hill, Deschambault, St-Joseph, and St-Hilaire, with indices of 2.77, 2.86, 3.13, 3.58, 4.11 and 4.88 respectively. The orchard richness also varied between seasons. Deschambault and Ile d'Orléans had the lowest richness indices in the spring (3.05; 2.66) and the highest in fall (4.16 and 4.29). Pooling all the orchards together, the spring collection had an higher level of richness (7.06) than the autumn collection (4.95) . Because the collected leaves came from unsprayed orchards, scab lesions were common on leaves and may have been a factor in the genera richness.

It was confirmed by Stadelmann and Schwinn (1976) that *V. inaequalis* increases the number of microorganisms present on apple leaves probably by influencing the host cell permeability, by excreting material which can be used by saprophytes or by higher transpiration which gives a more favourable relative humidity. Using cluster analysis, a dendogram was made to determine if the different orchards represent different fungal compositions, according to

the different genera (Fig. 5). When seasonal data for each orchard was pooled, the composition of each orchard appears to be fairly unique, since the first, second and third cluster occur at an Euclidean distance of 5.21, 7.73 and 9.29 respectively.

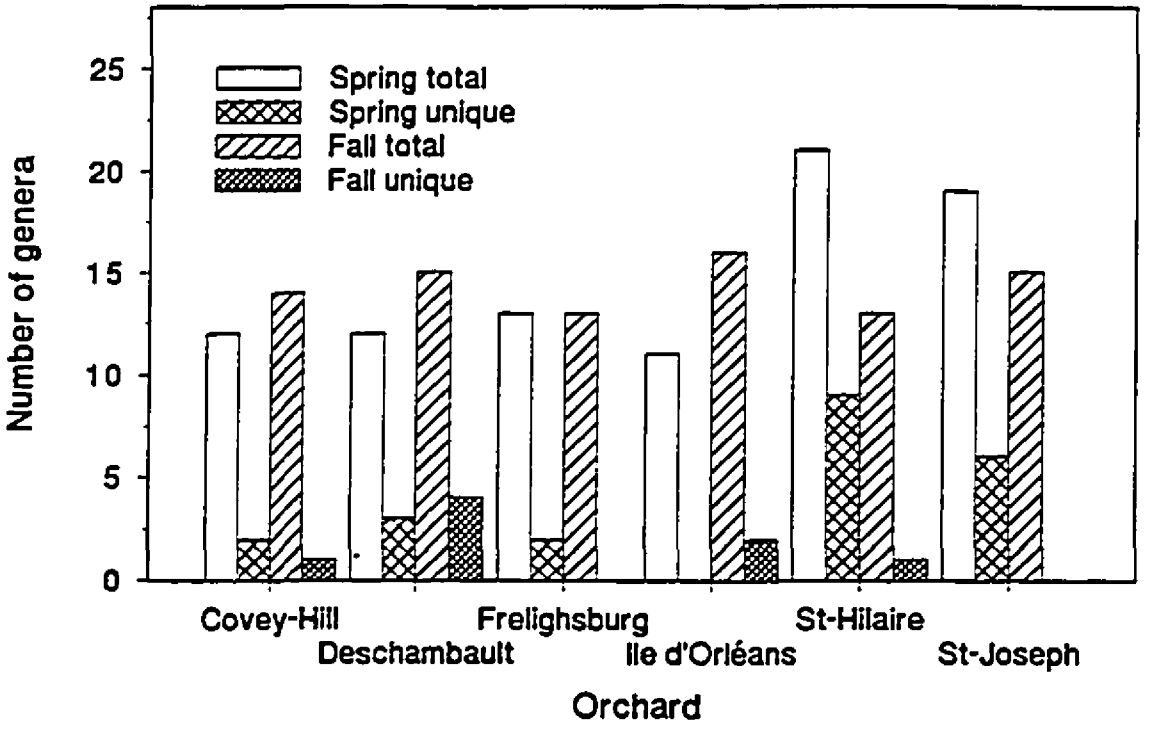

FIG. 4: Number of genera isolated from each orchard for the spring and fall

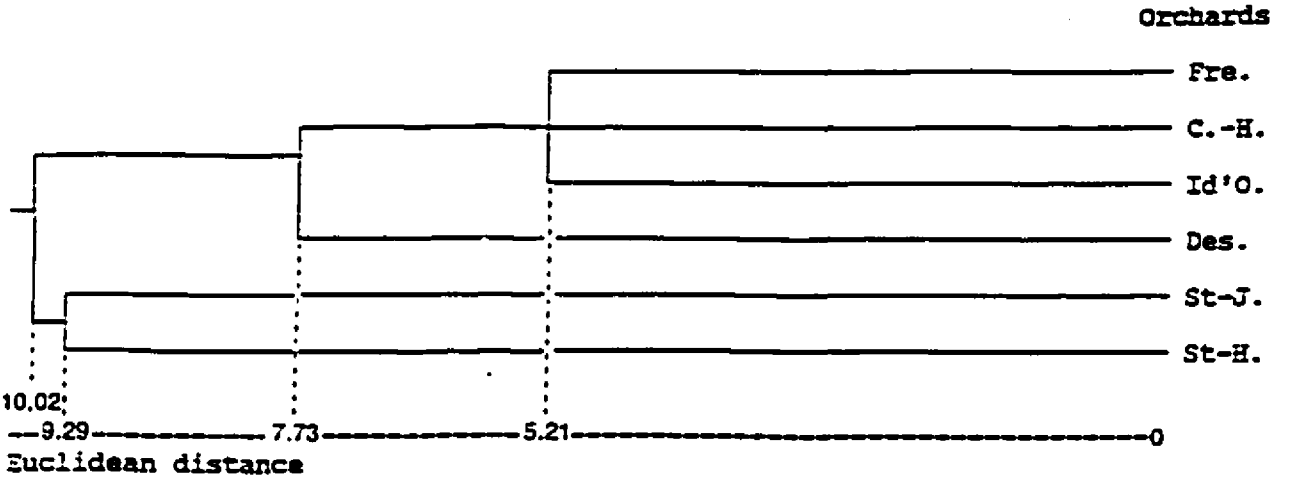

FIG. 5: Dendogram of fungal genera composition in apple leaves of six orchards in Québec. Data of the two seasons were pooled. Scale: each _ is equal to the distance of 0.135784. Refer to Figure 1 for orchard abbreviations.

Although the diversity (RI indices) among orchards were similar, they were not composed of the same genera. The two orchards with the highest index of richness (St-Joseph and St-Hilaire) were the most different from the other orchards, suggesting that the greater the number of genera, the greater the chance that they will be different

However, most of the commonly isolated fungi did not show seasonality, with most of the isolates present in both collections (Figs. 6 and 7). *Ceratosporella* was only isolated in the fall (Fig. 8). Isolates of *Epicoccum* and yeasts were equally divided between isolates obtain from both seasons and those only isolated in fall (Fig. 9). None of the isolates of *Trichoderma* were restricted to the fall collection (Fig. 10). This suggests that fungi colonizing the leaves by late fall may survive into the following spring. However, differences in distribution frequency can be affected by temporal succession of fungal communities in apple leaves as mentioned by Andrews and Kenerley (1978, 1979).

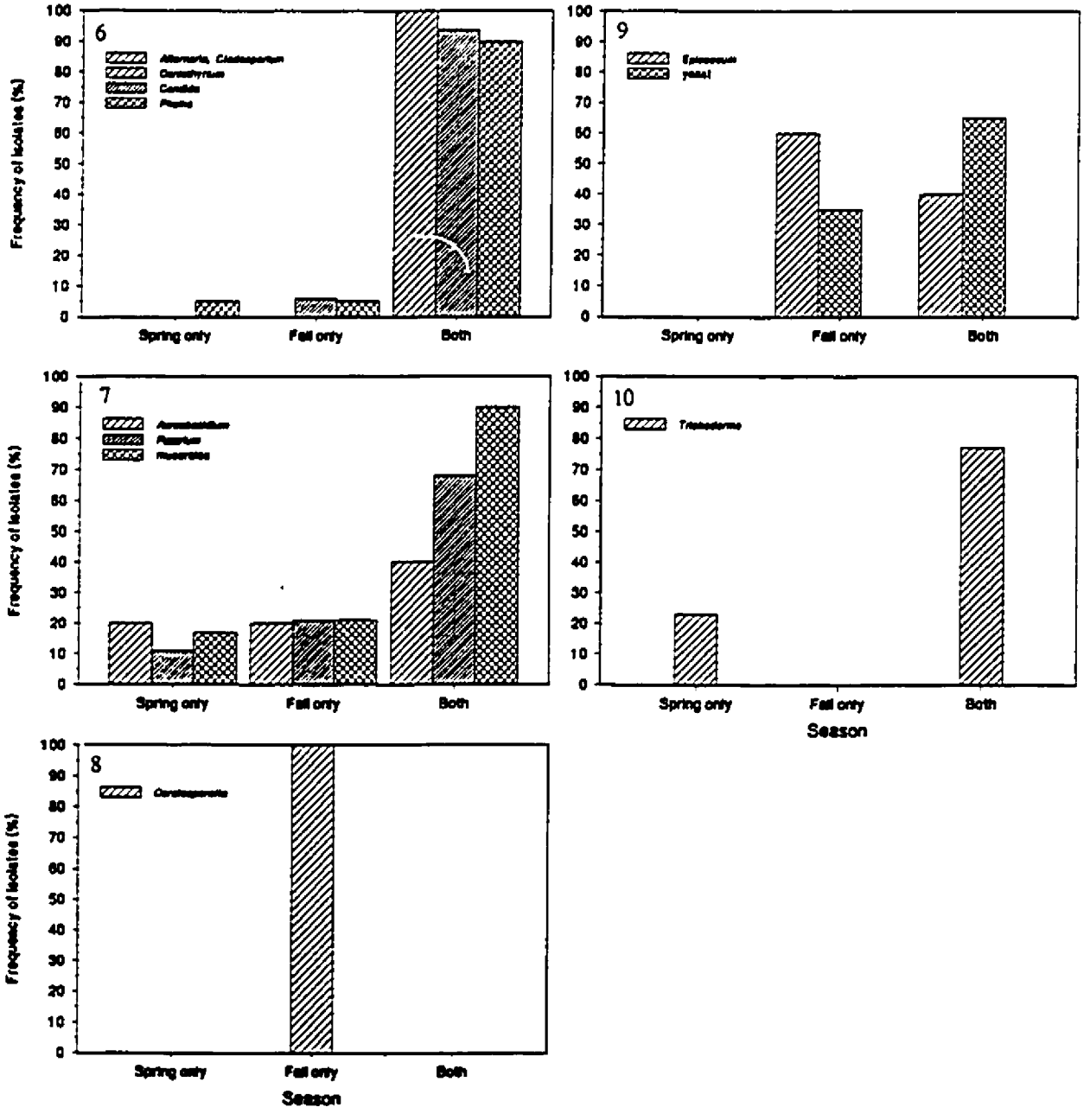

FIG. 6 to 10: Frequency of isolates of different genera according to their presence by season. Data among the different orchards were pooled . Only fungi that were present in more than one orchard for at least one season and isolated with a frequency of at least 2% are represented.

2.5 Conclusion

Our collections contained 24 new genera not previously reported from apple leaves. The genera *Bactrodesmium, Brachysporium, Cephalosporium, Ceratosporella, Chaetophoma, Chalara, Curvularia, Cylindrocarpon, Gilmaniella, Hendersonia, Humicola, Melanconium, Monilia, Mortierella, Mycogone, Papularia, Pyrenochaeta, Rhinotrichum, Rhizoctonia, Selenophoma, Sphaeropsis, Tubercularia, Ulocladium, Varicosporium* have not been isolated from apple green leaves or dead leaves by previous workers (Ross 1953; Simard et al., 1957; Andrews and Kenerley, 1978, 1979, 1980).

Our collection also contains 15 new genera which have never been reported on *Malus* Mill: *Cephalosporium, Cylindrocarpon, Hendersonia, Monilia, Pyrenochaeta, Selenophoma, Sphaeropsis, Tubercularia* and *Ulocladium* . (Farr et al., 1989; Jones and Aldwinkle, 1990)

This survey clearly demonstrated the large diversity of fungal species living in or on dead apple leaves. These fungi may be phylloplane inhabitants of living leaves, airborne colonizers of senescent leaves, or soil inhabitants. Cluster analysis confirmed our hypothesis that several sampling sites were necessary to provide a large diversity of genera since the composition of microbial community for each orchard was fairly unique. Sampling in only one orchard would had considerably reduced the number of different genera obtained.

Furthermore, this is the first complete study of the fungal communities present in several orchards in Quebec. One important finding of this survey is differences in the fungal composition among different orchards, suggesting that a potential antagonist might not have the same efficiency in every orchard due to differences in competition.

On the other hand, this large diversity should increase the chances of finding antagonists that interfere with the overwintering of *Venturia inaequalis* . The fungi isolated from this study will be tested for their capacity to inhibit the overwintering stage of *V.inaequalis*.

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CONNECTING TEXT 1

Once the collection of potential antagonists was done, considering the large number of isolates collected, the need to screen the organisms *in vitro* according to a defined criteria was needed. The criteria was based on the objective we want to achieve. To control apple scab, our approach was to hasten the microbial decomposition of leaves and destroy the leaf substrate in which pseudothecia are formed. Even if the antagonistic effect of a biocontrol agent using the competition seems to play a relatively minor role in the organization of natural phylloplane communities, we believe that because of the life cycle of *Venturia inaequalis* this mode of action becomes a promissing perspective. Therefore, we searched for isolates from the fallen leaves capable of growing at low temperatures that may be adapted to leaf litter conditions in Quebec during the time pseudothecia are formed. Furthermore, working with fungi from apple leaf litter permits us to find not only microorganisms from the living leaves but also fungi originating from the soil surface and thus, probable decomposers.

After making the collection of fungi, the different isolates were thus evaluated for their degradative enzymes and for apple leaf decomposition ability in relation to ascospores production *in vitro.*

3. EVALUATION OF FUNGAL ISOLATES FOR THEIR GROWTH ON MEDIA, ABILITY TO DECOMPOSE LEAVES AND INHIBITION OF ASCOSPORE FORMATION OF *VENTURIA INAEQUALIS*.

3.1 Abstract

With the hope of finding biocontrol agent against apple scab, Bernier *et al.* (1995) made a collection of fungi from dead apple leaves. These fungi were examined for their capacity to decompose apple leaves and inhibit ascospore production. The capacity of these isolates to grow on cellulose (CMC), pectin (PA) and chitin (CA) agar and the effect of the fungi on leaf rheology were also studied. Of all the fungi tested, 77% of the isolates grew better on at least one tested culture media (CA, PA, CMC) than on V8. Of these fungi, 28% grew on CMC, while only 2% grew on PA. Twenty-three percent of the isolates grew better on CA-CMC than on V8 and 27% grew better on CA-PA-CMC. Of the 181 isolates tested for leaf rheology, 29% significantly degraded leaves compared with non-inoculated controls. Twenty-six percent of fungal isolates tested inhibited ascospore production of *Venturia inaequalis* , the range being between 87% and 100%. One-third of the significant fungi markedly decomposed the leaves according to the visual observation of the suspension after bubbling. No correlation was found between the different parameters measured.

3.2 Introduction

Venturia inaequalis (Cke) Wint. is an ascomycete which is responsable for apple scab, one of the most important disease on stone and pome fruit around the world, . This disease has been controlled by fungicides for several decades, however this method is loosing its efficacy. The pathogen is showing increasing resistance to different fungicides such as benomyl, dodine and fenarimol (Jones, 1981; Carisse and Pelletier, 1994). Moreover, because of the environmental impacts of these chemicals, there is an increased interest to find alternatives to the use of fungicides. Cultural control, host resistance and biological control are options to be considered in the managament of apple scab.

One alternative to control the disease is the management of the fallen apple leaves, since the pathogen overwinters in apple leaf litter. It was demonstrated by several techniques (leaf shredding, nitrogen enrichment, urea applications) that if leaf decomposition is enhanced, ascospore production by *V. inaequalis* is reduced. (Hirst and Stedman, 1962; Miller and Rich, 1968; Ross and Burchill, 1968; MacHardy, 1990). In addition, it was demonstrated that there is a relationship between the potential ascospore dose present in the orchard and the severity of scab during the season (MacHardy, 1990). Unfortunately, scab management programs assume that the amount of primary inoculum (ascospore production) is always sufficient to cause an unacceptable level of apple scab if the recommended fungicide schedule is not followed. Therefore it is important to work on the perfect stage of the pathogen to reduce the primary infection (MacHardy and Jeger, 1983).

Studies have been conducted on different ways of controling the disease by leaf management. Heye and Andrews (1983) observed that leaves inoculated

with the fungus *Athelia bombacina*, a leaf inhabitant, became softer and decomposed faster, supporting less development of pseudothecia.

Bernier et al. (1995) collected fungi from dead apple leaves which were capable of growing at low temperatures and that may be adapted to leaf litter conditions during the time pseudothecia are formed. Because the saprophytic stage of *Venturia inaequalis* occurs in leaf litter, this substrate becomes a critical element of survival. Because the saprophytic stage is also long lasting, competition from other organisms for nutrients or for the substrate during this phase is likely to occur. We hypothetized that leaf decomposition is a measure of this competition.

In this paper, we examined the capacity of different isolates to decompose apple leaves and inhibit ascospore formation. We also investigated the ability of the isolates to degrade cellulose and pectin (plant cell wall components) and chitin (a fungal cell wall component). Finally, we examined the correlation between these isolate phenotypes in relation to biological control.

3.3 Materials and methods

3.3.1 Growth of fungal isolates on amended media

To verify if the isolated fungi have a chitinolytic, pectolytic, or cellulolytic activity, fungal growth on chitin agar (CA), pectate agar (PA) and carboxymethylcellulose agar (CMC) was measured (Figure 11).

Growth rate (mm of growth/day) on V-8 served as the positive control. Water agar served (15g/L) as the negative control. The different amended media all contained the following ingredients: 0.5g/L; $MgSO_4.7H_2O$: 0.5g/L; NH_4NO_3: 1g/L; KH_2PO_4: 1g/L; agar: 20g/L. Growth on amended media (CA (1.5g/L of crab shell chitin), PA (0.5g/L of sodium polygalacturonate) and CMC (15g/L of carboxymethylcellulose)) was measured and compared to the controls to evaluate if the fungi have any ability to grow on the different media.

Plugs of 7 mm diameter from half-strength V-8 agar cultures served as inoculum. The plugs were taken from the periphery of the colony. There was one plug per dish, 4 replicates per fungus, and 5 media per fungus were used. The test was conducted at 15°C. The diameter of the colony was measured daily for the fast growers and every 2 to 8 days for the other ones. The measurments were taken until the fungus reached the edge of the plate for at least one of the 5 media tested. The experiment was done twice. Effect of amended media on fungal isolates was tested using analysis of variance and the difference among growth rates on the media was located using the LSD test.

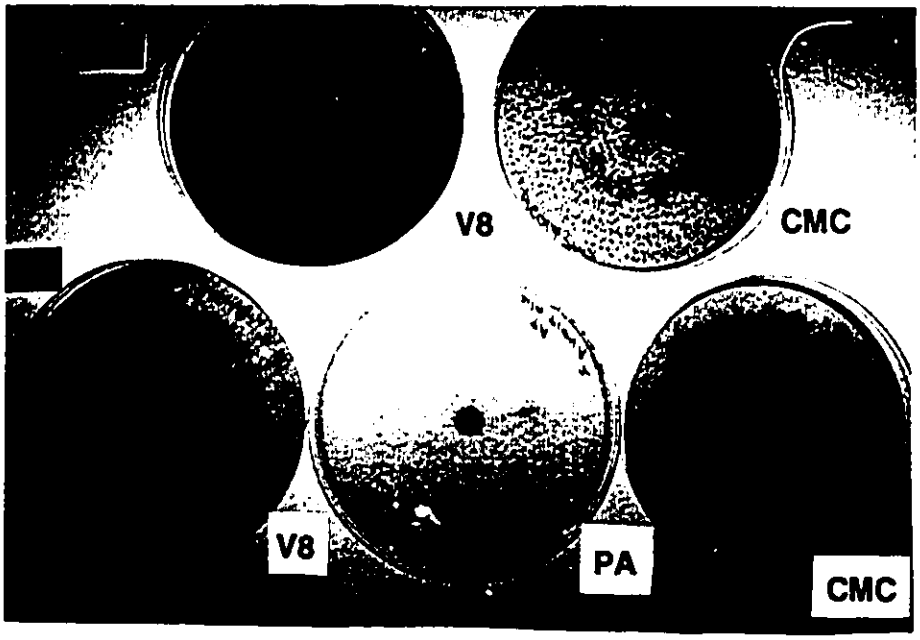

FIG. 11: Example of fungal growth on media amended with different carbon sources (V_8, PA: pectate agar, CMC: cellulose).

3.3.2 Test of leaf decomposition

Apple leaves (cv. McIntosh) still remaining in trees were collected in mid October and at the begining of November at the Agriculture and Agri-Food Canada, experimental farm in Frelighsburg (Quebec). Plastic petri dishes were filled with 30 g of sterile silica and 8 ml sterile distilled water to provide moisture and physical support for the leaves. The discs were surface sterilized by five successive washings of one minute in 1% sodium hypochlorite, followed by five other washings of two minutes in sterile distilled water. The purpose of this step was to eliminate the external contaminants that could compete with the tested isolates and to avoid a decomposition caused by a contaminant.

The leaf discs were then placed in the dish and inoculated with a suspension of each isolates. For each fungus, four petri plates with 3 discs/plate were used. Each suspension was prepared from one culture on V-8 agar containing the fungus covering the whole surface of the plate, otherwise, two or more plates were used. The inoculum was placed in a sterile plastic stomacher bag with 50 ml of sterile distilled water. The suspension was obtained by homogenizing the bag contents with a Stomacher™ laboratory blender (Seward Medical, London, U.K.). A sterile Eppendorf repeater type syringe was used to inoculate the upper surface of disc with 50 µl of the suspension. Non-inoculated discs were used as control. The plates were kept at 15°C for 100 days (Hankin, 1976) and the whole experiment was done twice.

The degree of decomposition was evaluated with a rheology test which measures the strength required by an anvil to perforate the leaf (Figure 12). The leaf thickness was measured with a micrometer and then the disks were placed firmly between two Plexiglas plates held together with paper clips. The Plexiglas plates contained a hole in the middle just large enough to let a needle of 6.35 mm diameter penetrate. The measurements of thickness and strength were taken at the same place on the disk, and major veins were avoided. The Plexiglas plates holding the discs were then placed in the slot of the Instron® penetrometer (Instron Corp. model 4201. Mass. USA). A 50-Newton cell was used to take the readings and the penetrometer needle speed was set at 100 mm/min. The strength that caused rupture (peak load) was recorded. The strength needed for the perforation was divided by the thickness of the leaf disc to correct for leaf thickness. Because of the large number of fungal isolates to be tested, the experiment was done in four series of inoculations, each one with its own control.

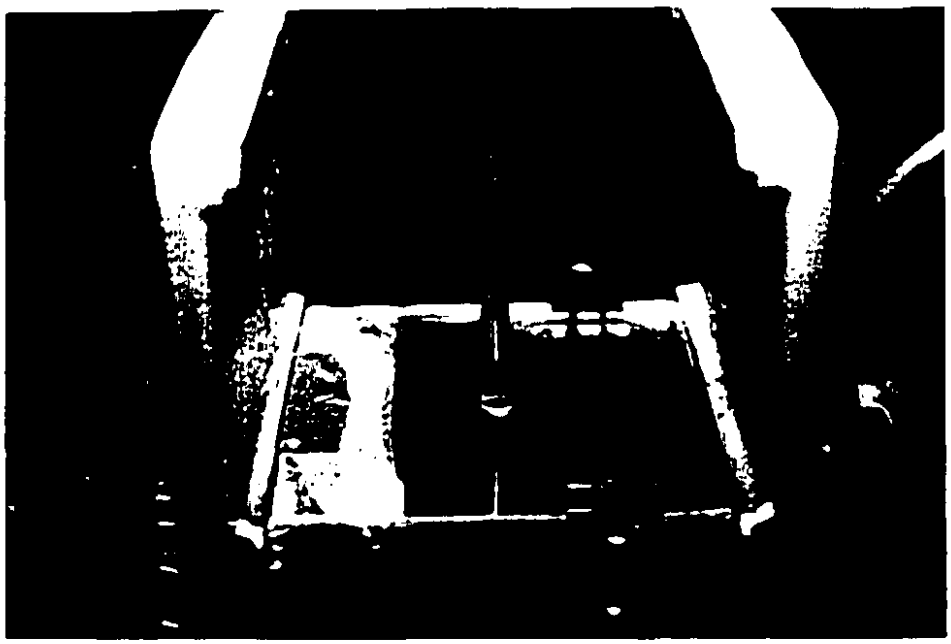

FIG. 12: Picture of the penetrometer anvil perforating a leaf disc held in two plexiglass sheets.

3.3.3 Evaluation of fungi to inhibit ascospore production

Leaf material - Non infected apple leaves were collected from McIntosh trees in the beginning of September 1994 at Frelighsburg. Discs of 2.7 cm diameter were cut using a home-made cork borer. The major veins were avoided to ensure uniformity of the leaf tissue. The leaf discs were put in a mustard type glass jar and sterilized by irradiation at the Centre de Recherches et de Développements en Alimentation (CRDA) in St-Hyacinthe, Québec. The irradiation process lasted was 10 hours and the jar and its contents were submitted to 40 kGy (4Mrad) of gamma radiation.

Incubation chamber - Glass jars with screw cap were used as incubation chambers. The jars were filled with 100 ml of perlite and 50 ml of distilled water, and were autoclaved for 20 minutes at 121°C. After a cooling period, two leaf discs (abaxial surface up) were put on the perlite.

Inoculation of Venturia inaequalis - Six different isolates grown on V8 agar were used to prepare the fungal suspension: 1An1.3, 9A1.1, 15A1.1, 18b2.2, 26a3.3 and 15d2.1 in a 1:1:1:1:1:1 ratio. The isolates were collected from numerous orchards in Quebec. The suspension, previously homogenized with 100 ml of sterile distilled water in a stomacher and then filtered through a cheese cloth, was inoculated by putting 100 µl on each disc, two discs per jar and four jars per fungus, using Eppendorf™ repeater type Combitips™ syringes. The jars were then incubated for 21 days in darkness at room temperature.

Inoculation of the antagonists - The antagonists were prepared by growing them on V8 agar. After the initial 3 weeks of incubation, suspensions of the different fungi were prepared by adding the contents of a plate in 100 ml of sterile distilled water in a sterile plastic bag used for the stomacher. The fungi were homogenized 30 seconds and 100 µl was inoculated to each disc, two discs per jar and four jars per fungi, using Eppendorf™ repeater type Combitips™ syringes. Controls with *Venturia* only and *V. inaequalis* with agar were also kept. The jars were incubated at room temperature, 4°C and 10°C following the protocol established by Philion (1995) except that the last 2 weeks at 10°C were replaced by two weeks incubation at room temperature (Figure 13, 14).

FIG. 13: Incubation chamber (glass jar) containing two leaf discs laying on perlite inoculated with *Venturia inaequalis*.

Ascospore extraction- After four months of incubation, the ascospore production was measured by using a home-made bubbler previously developed (Philion, 1995) to permit the spore ejection (Figure 15). The procedures used were the same as those described by Philion (1995). The air flow was set around 0.1 scfm in Falcon Blue centrifuge tubes of 50 ml (Beckton Dickinston #20948) containing 20 ml of water and the two leaf discs from each jar. The bubbling was run for 1 hour. Then the leaf discs were removed and a few drops of Lugol's iodine were added. The suspensions were centrifuged 10 minutes at 3800 rpm and the pellet was saved with a remaining 5 ml of suspension to avoid pellet disturbance. The tube contents were shaken and the ascospores were counted using an haemacytometer (Fuch Rosenthal; C. A. Hausser and Son, Max Levy Inc. USA). A conversion factor was used to record the number of ascospores per cm^2 of leaves. Starting with a screened volume of 6.4 mm^3 (or 6.4 µl) (2 haemacytometer surfaces, 16 squares/surface, and each square being 1 mm^2 and 0.2 mm deep). To calculate the number of spores/ml, we multiplied by 156.25. The two leaf discs represent together a surface of 11.45 cm^2, and the spore pellet was repuspended in 5 ml. To calculate the number of ascospores/cm^2, the number of counted ascospores were multipled by 68.23 (156.25 x 5/11.45). Leaf disc decomposition was estimated by visual observation of the suspension color. Since the more the disk is decomposed, darker is the suspension (Philion, 1995), score of 0 to 4 were used, where 0 was a clear suspension corresponding to no decomposition.

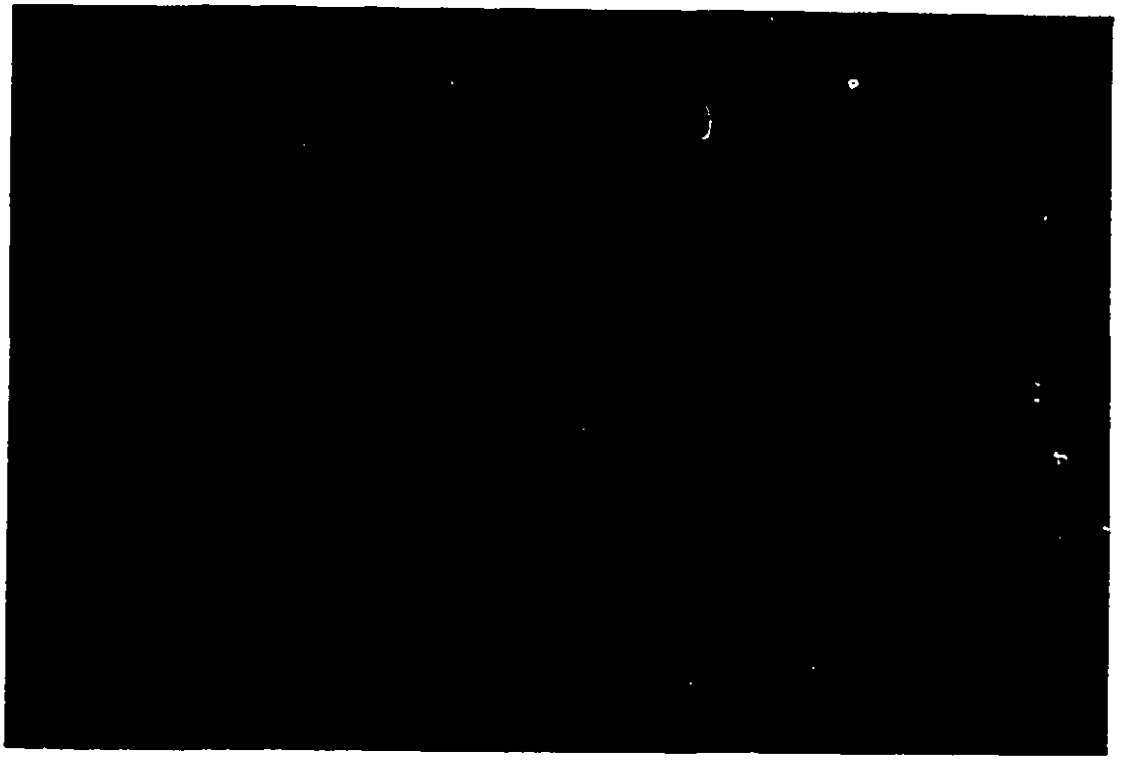

FIG. 14: Squash mount of *Venturia inaequalis* pseudothecium containing ascospores after 4 months of incubation.

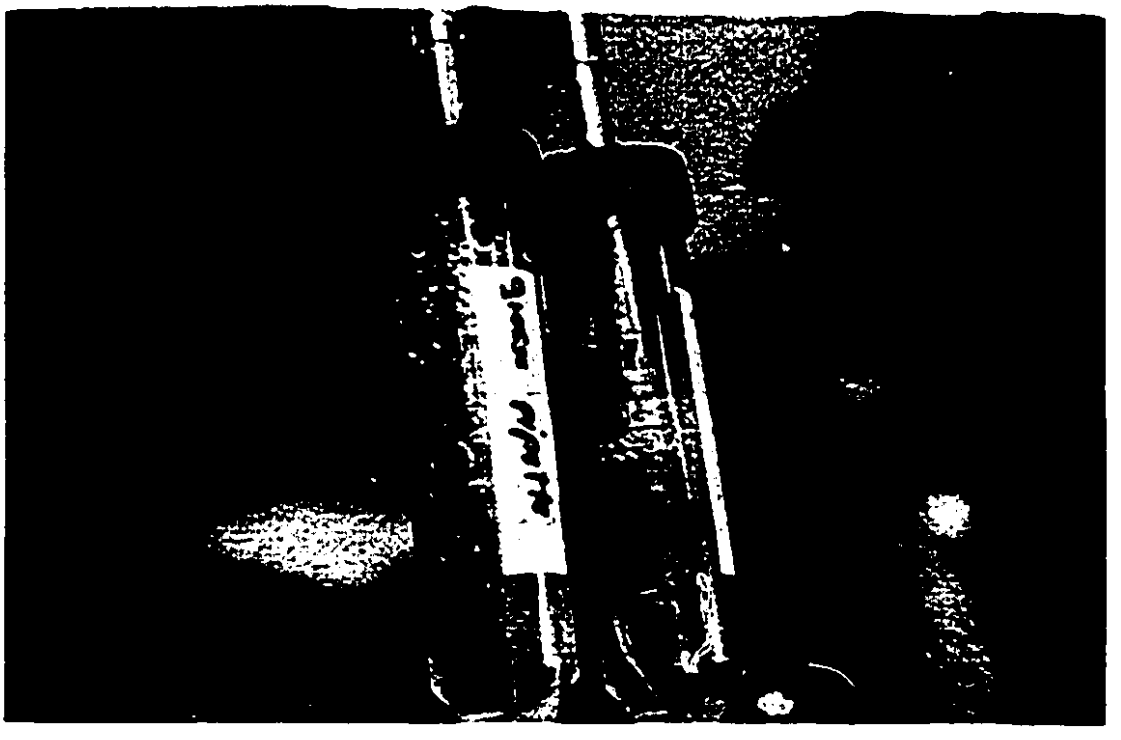

FIG. 15: Picture of the ascospore extractor with leaf discs being bubbled.

Statistical analysis- For the three experiments, a one tailed T test at P=0.05 level using ANOVA was conducted to determine if the tested media had a significant effect on fungal growth, leaf rheology and ascospore production. To stabilize the variance, the mean of rheology used was the strength needed for the leaf penetration divided by the thickness of the leaf. For the experiment on inhibition of ascospore production, the data were transformed with $Y=Log(x+1)$ and the percentage of inhibition was calculated using 10^{x+1}. Spearman tests of correlation were conducted to determine whether the effect of media on fungal growth, effect of fungal isolates on leaf rheology and effect of fungal isolates on ascospore production were correlated. Non-parametric Spearman test of correlation was used to determine if ascospore production was correlated with the estimated decomposition of the bubbled leaf discs by the visual observation of the suspension scored according to a determined scale.

3.4 Results

The growth on different carbon sources was evaluated by testing the fungal isolates on different culture media amended with chitin, pectate or carboxymethylcellulose. The growth rate of the isolates on these media was compared to the growth rate on the control, V8 agar (Table III). Water agar (WA) was used as a negative control to observe the growth on a media without a carbon source thus using its own reserves. However, the growth rates were not compared to this medium, since the fungi often produced thin but expansive hyphae.

ANOVA tests indicated that the different media had a significant effect on fungal growth at the level P=0.05. When looking at all fungal isolates tested, 77% were able to grow on at least one of the tested culture media (CA, PA, CMC) (Figure 16). These fungi had an equal or a significantly higher growth rate on these media than on the V-8 control. Of these isolates, 28% grew on CMC while only 2% grew on PA. Twenty-three percent grew on both CA and CMC and 27% grew on CA, PA and CMC (Figure 16). We did not consider the fact that the different amended media did not have the same amount of carbon. When refering at the recipes, CA (1.5g/L) had three times more carbon than PA (0.5g/L) and PA had fourty times less carbon than CMC (20g/L), this difference could have an effect on the relative growth amongst the media. The fact that the frequency of fungi growing on CMC is the highest may be related to the high carbon quantity present in the media. Furthermore, because crab shell chitin flakes were used instead of colloidal chitin (dissolved in acid), the chitin was potentially less available to the tested fungi.

TABLE III: Effect of amended culture media on growth of fungal isolates.

	Growth rate on media (a)						Growth rate on media (a)				
Isolates	V8 (b)	WA (c)	CA (d)	PA (e)	CMC (f)	*Isolates*	V8 (b)	WA (c)	CA (d)	PA (e)	CMC (f)
A144A	4.51ab	3.82bc	4.94a	1.95d	2.82cd	P114A	2.08a	1.82b	2.13a	1.73b	2.17a
A152A	6.69a	4.92b	6.92a	6.68a	6.85a	P115A	4.42a	3.13b	4.09a	4.45a	4.03a
A154A	4.93a	3.82c	4.85ab	4.67b	4.78ab	P118A	4.64a	3.45bc	2.68c	3.49b	3.6b
P6A	1.62a	1.73a	1.29b	0.85c	1.39b	P121A	7.51a	3.91c	5.68b	6.17b	5.86b
P7B	4.38a	3.70ab	3.20b	2.24c	4.30a	P124A	4.41a	2.80c	1.29d	1.81d	3.71b
P9D	14.3a	6.29d	12.55bc	12.04c	13.11b	P126A	5.35ab	4.01c	3.73c	5.21b	6.27a
P10C	13.08a	6.38d	10.87b	10.14c	12.41a	P132A	12.13a	5.68c	7.28b	6.57b	7.18b
P12A	8.71a	3.81c	3.73c	4.35c	7.37b	P134A	12.22a	7.17d	8.13c	11.18b	11.11b
P13B	6.65a	1.52d	2.94c	2.43c	5.39b	P135A	8.59a	4.61d	7.40ab	6.08bc	4.90cd
P14B	6.49a	0.81d	2.12c	1.79c	4.98b	P136A	1.70a	0.92b	1.32ab	1.29ab	1.49a
P15A	7.12a	3.24d	3.75c	3.75c	4.97b	P138A	2.69ab	2.78a	1.53d	2.54b	2.14c
P18A	0.88a	0.94a	0.75a	0.46a	0.54a	P143A	4.88a	2.26c	3.73b	2.73c	2.5c
P25A	3.20a	2.43c	2.95a	2.71bc	2.79b	P144A	14.67a	8.95d	10.28b	9.23c	8.04e
P27A	2.13ab	2.32a	1.75bc	1.79bc	1.58c	P145A	11.41a	7.58b	7.83b	8.03b	7.68b
P29A	2.91a	2.71b	2.83ab	2.96a	2.17c	P146A	7.39a	2.57d	4.91b	2.92d	3.92c
P32A	3.93a	2.32b	2.62b	2.64b	1.62c	P147B	9.16a	5.48c	7.84b	5.61c	5.78c
P40A	4.34a	2.48d	2.65c	2.05e	3.78b	P149A	4.05a	0.00b	0.12b	0.79b	0.15b
P42A	4.07a	3.37c	3.13d	3.27cd	3.65b	P153A	5.34a	5.38a	4.55bc	4.10c	4.89ab
P43A	3.81a	3.46a	3.26a	3.46a	3.64a	P155A	3.85a	2.36d	3.48b	3.24c	3.48b
P46A	3.00a	2.48cd	2.86ab	2.36d	2.66bc	P156A	1.80a	0.8bc	0.95b	0.51c	1.57a
P47A	4.24a	3.80b	3.31c	2.45d	2.29d	P163A	2.84a	2.72ab	1.46c	2.60ab	2.40b
P48A	8.02a	0.00c	5.82b	4.42b	1.41c	P173A	13.31a	8.26d	10.25c	12.04b	11.96c
P49A	5.15a	4.47bc	4.62b	4.12c	4.82ab	P176A	3.40a	2.51b	2.46b	2.05c	2.56b
P50A	4.36a	3.78b	2.92c	2.53d	4.18a	P179A	7.07a	2.98c	3.18c	3.66b	2.53d
P57A	4.60a	3.21d	4.66a	3.98c	4.41b	P181A	1.66a	0.89c	1.46ab	1.25b	1.59a
P58A	1.89a	1.78ab	1.72b	0.60c	1.72b	P183A	1.96a	0.98d	1.71b	1.26c	0.88d
P65A	4.11a	2.92b	1.52c	2.06c	3.42b	P187A	8.20a	1.54e	4.13d	4.83c	6.88b
P66A	2.32a	1.87ab	2.18a	1.52b	2.32a	A50A	8.72a	3.43d	7.60b	3.89c	3.97c
P77A	5.76a	3.24d	5.33b	3.26d	4.44c	A53B	9.48a	5.14e	7.73c	8.40b	7.22d
P79A	1.78a	0.91c	0.80c	0.51d	1.44b	A54A	5.52a	1.10e	4.76b	2.74d	3.31c
P80A	3.87a	2.76c	2.04e	2.34d	3.50b	A66A	3.79a	2.41d	3.20b	2.76c	3.25b
P84A	2.92a	2.66b	2.68b	2.90a	2.27c	A74A	5.33a	0.99c	5.09a	4.50b	4.42b
P86A	4.61a	4.50a	2.67c	2.35d	4.08b	A74B	5.28a	4.93a	4.24b	4.98a	3.21c
P89A	3.83a	2.93d	3.43b	2.69e	3.18c	A79A	15.86a	2.22c	4.76c	4.21c	10.49b
P92A	3.67a	2.76d	2.97c	2.90cd	3.31c	A80A	2.77a	0.84e	2.37b	1.66d	2.01c
P94A	3.54a	2.76b	2.15c	2.68b	3.34a	A83A	15.50a	6.14e	12.65b	11.15c	9.44d
P95A	1.69a	1.21d	1.50b	1.33c	1.64a	A107A	4.16a	3.38d	3.73b	3.18e	3.55c
P97A	3.57a	2.65c	2.41d	2.57c	3.19b	A111A	4.44a	3.69c	3.94b	3.25d	3.84b
P102B	3.28a	2.49d	3.13b	2.62d	2.92c	A112A	4.51a	3.90b	3.97b	3.19c	3.82b
P104A	10.22a	0.38e	4.86b	3.45c	1.46d	A150A	4.66a	2.32d	4.32b	3.90c	4.39b
P105A	8.89a	1.93e	4.04c	3.53d	8.44b	A151A	6.87a	4.99c	6.65a	5.63b	5.03c
P107A	5.79a	4.36c	5.17b	5.01b	5.74a	A155A	5.16a	3.32e	4.92b	4.07d	4.42c
P108A	4.31a	2.59c	2.27d	2.23d	3.96b	A156A	2.49a	0.46e	1.81c	2.17b	1.32d
P110A	9.42a	8.08b	7.67bc	7.24cd	7.05d	A157A	4.80a	3.39c	4.25b	3.39c	4.30b

(a): mean of growth divided by the number of tested days (mm/day)

(b) -(f): b: control, c: water agar, d: chitin agar, e: pectate agar, f: carboxymethylcellulose agar.

g:Values within the same row followed by the same letter are not significantly different according to LSD test ($P \leq 0.05$)

TABLE III: Continued

	Growth rate on media (a)						Growth rate on media (a)				
Isolates	V8 (b)	WA (c)	CA (d)	PA (e)	CMC (f)	*Isolates*	V8 (b)	WA (c)	CA (d)	PA (e)	CMC (f)
P2B	4.23abg	3.55b	2.82c	1.61d	4.59a	A91A	4.35c	4.94b	5.70a	2.89d	4.49c
P4A	2.29ab	1.72c	1.60c	2.23b	2.69a	A136B	5.40b	5.33b	5.84a	3.41a	4.55c
P5A	1.12c	2.13b	2.44b	2.33b	3.23a	A148A	5.23ab	3.73d	5.08b	4.22c	5.38a
P10A	13.64b	9.59e	14.98a	12.55c	11.56d	A153A	5.29ab	5.10b	5.54a	3.82a	4.53c
P35A	2.13b	2.06bc	2.41a	1.93c	1.98bc	A159A	2.78b	0.81d	3.08a	2.57c	2.80b
P39C	7.14a	5.18c	7.11a	6.11b	6.83	A162A	2.96a	1.72c	2.59b	2.51b	3.12a
P41A	4.95b	4.23c	5.19a	4.20c	5.18a	P8A	13.42a	5.87c	11.37b	10.71b	12.80a
P45A	4.50c	4.21d	4.80d	4.52c	5.07a	P11A	13.17a	9.73c	12.44b	12.54b	13.55a
P60A	4.19a	3.35d	3.97b	3.78c	4.08ab	P20A	3.12a	2.66b	2.97a	2.45b	3.08a
P61B	4.15b	2.81c	2.82c	2.45c	5.49a	P26A	1.52a	0.87b	1.50a	1.00b	1.05b
P63A	4.31b	3.55b	3.91b	3.66b	5.40a	P28A	1.14a	1.13ab	0.97ab	0.80b	1.27a
P64A	3.98a	3.89a	4.20a	2.53b	3.76a	P53A	1.47abc	1.75a	1.35bc	1.26c	1.58ab
P68A	2.44a	1.85b	1.53b	2.44a	2.70a	P55A	5.13a	3.99b	4.23b	2.35c	5.54a
P72A	2.61bc	2.90ab	3.08a	1.87d	2.35c	P62A	4.09a	3.25b	3.90a	2.78c	3.98a
P75A	2.59b	3.15a	3.05a	3.04a	2.64b	P69A	2.72a	2.10b	2.43ab	1.21c	2.81a
P78A	3.72b	1.07e	2.44d	2.80c	4.48a	P71A	2.61a	2.01c	2.60a	2.29b	2.63a
P83A	0.86c	1.77a	1.26b	1.28b	1.13bc	P73A	5.87a	1.97c	3.49b	3.70b	5.64a
P84A	5.73b	5.11c	3.39d	6.04a	6.00a	P74A	6.98a	5.17b	5.55b	6.37a	7.07a
P85A	4.94b	4.27c	5.75a	5.03b	5.63a	P76A	2.12a	1.93b	2.14a	1.47c	2.04a
P87A	1.62a	1.04b	1.63a	1.01b	1.80a	P81A	2.04a	1.81a	0.87b	0.86b	2.01a
P103A	2.31c	2.96bc	3.55b	3.12bc	4.39a	P82A	2.85a	1.78a	1.39a	1.25a	1.87a
P106A	4.69b	4.36c	2.95d	2.77d	5.27a	P88A	1.70a	0.85c	1.61a	1.34b	1.68a
P111A	4.33b	4.49c	3.02d	2.81d	4.93a	P93A	1.98a	1.39d	1.81b	1.57c	2.03a
P116A	6.13b	4.41c	6.01b	6.05b	6.43a	P98C	1.73a	1.02c	1.39b	0.83d	1.72a
P117A	3.34c	3.90b	4.64a	4.33ab	4.41a	P101A	3.50a	2.38c	3.47a	2.76b	2.91b
P120A	6.64b	6.13c	7.07a	6.51b	7.13a	P109A	6.13a	4.98c	5.94a	5.43b	6.00a
P122A	4.73b	3.10d	5.03a	4.28c	4.45c	P113A	6.53a	5.82c	6.28ab	5.97bc	6.35ab
P123A	4.67b	4.16c	4.84ab	4.26c	5.02a	P119A	7.62a	4.89bc	4.33c	5.65b	7.74a
P125A	3.25c	3.09d	3.38b	3.00d	3.50a	P127A	4.26ab	3.11d	4.47a	4.08b	3.68c
P141A	1.38b	1.22bc	1.82a	1.13c	1.62a	P129A	4.45a	4.32a	3.00b	2.96b	4.60a
P150B	15.08b	11.50c	15.86ab	15.22b	16.16a	P130A	2.03a	1.44b	1.16c	0.93d	2.02a
P151A	2.51b	1.49c	1.95bc	1.43c	3.25a	P140A	6.11a	2.58c	4.08b	2.87c	5.72a
P158A	2.04a	1.63b	1.71b	1.63b	2.31a	P142A	1.86ab	1.98a	1.92a	1.77ab	1.62b
P178A	2.59b	3.40a	2.68b	2.34c	3.40a	P174A	3.37a	2.54d	3.28ab	3.22b	3.07c
P185A	1.54a	1.04c	1.23b	1.01c	1.63a	P177A	10.71a	3.40c	11.07a	8.83b	10.28a
P186A	5.58b	2.93c	6.42a	6.05ab	5.79b	P184A	11.40a	6.31c	11.80a	9.08b	11.63a
P188A	8.87d	4.64e	11.66c	12.82b	14.22a	A9A	12.40a	8.32b	12.21a	12.43a	12.02a
P189A	11.86b	9.41d	12.17b	11.30c	12.91a	A45A	4.17ab	1.32d	4.04b	2.62c	4.42a
P190A	9.26b	3.88c	12.24a	11.63a	12.78a	A49A	1.85a	1.38b	1.85a	1.84a	1.87a
P195A	11.93b	7.49c	15.00a	14.08a	15.62a	A55A	6.92a	5.57c	6.62ab	2.85d	6.20bc
P196B	12.41b	3.52c	14.69a	14.29a	15.45a	A62A	6.36a	5.88b	6.40a	2.86d	4.84c
A43A	5.84b	3.57e	5.48c	4.58d	6.14a	A69A	5.68a	4.61c	5.48b	4.51c	5.74a
A44B	4.43b	3.43e	4.09c	3.95d	4.69a	A89A	4.93a	4.02c	4.64ab	4.30bc	4.94a
A57B	3.14b	2.68c	3.19ab	2.65c	3.21a	A95A	3.19a	2.55b	3.23a	2.56b	3.21a
A70A	8.78b	5.60c	9.32a	9.24a	8.82b	A97B	6.05a	4.62c	5.82a	5.08b	5.95a
A72A	11.40b	8.55c	12.32a	12.28a	12.61a	A113A	2.88a	1.53b	2.81a	2.73a	2.64a

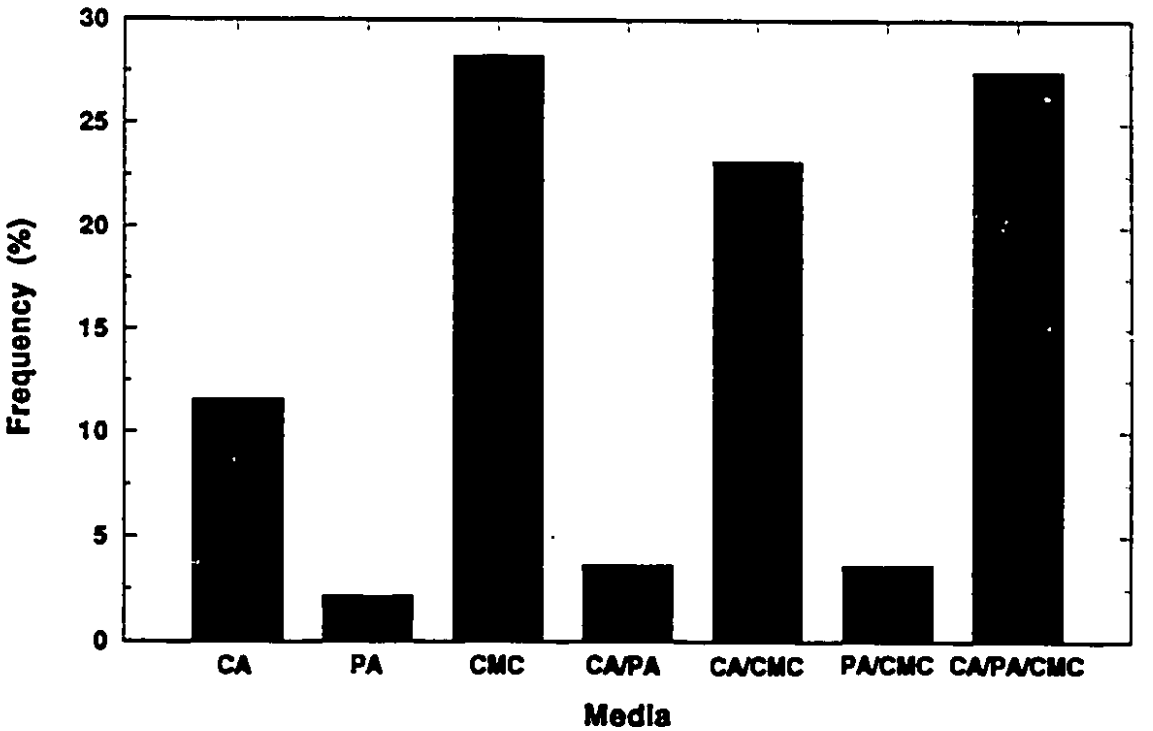

FIG. 16: Frequencies of the fungal isolates according to their growth on different culture media. Presented isolates grew equally or better on specified media than on the control media (V8). CA: chitin agar; PA: pectate agar and CMC: carboxymethylcellulose agar.

Out of the 181 isolates tested for leaf rheology, 30% of them significantly degraded leaves as compared to non-inoculated control (Table IV). Fungi with significant effect ($P \leq 0.05$) on leaf rheology decomposed leaves from 11% to 63%. Four controls were used in this experiment, one for each series of inoculation. Because each control had a different level of decomposition (3.3 N/mm to 8.7 N/mm), the degree of decomposition only was not enough to conclude a significant degradation since isolates having the same mean were not necessarely significantly different from the corresponding control.

Fungal isolates were also tested for the inhibition of ascospore production of *Venturia inaequalis* (Table V). Among the 118 fungi tested, 32% significantly inhibited ascospore production, the range being between 88% and 100%. For those which significantly reduced the ascospore production, the visual observation of the suspension after the bubbling, 33% of those isolates showed a marked decomposition (more than score 3). These decomposed suspensions were diluted 1.5 to 5 times to make the spore counts on the haemacytometer background. Isolates showing a negative percentage of inhibition had stimulated ascospore production.

TABLE IV: Effect of fungal isolates on leaf rheology of apple leaf discs.

FIRST SET				SECOND SET			
Isolates	Mean (a)	% (b)	T Test (c)	Isolates	Mean (a)	% (b)	T Test (c)
P180A	3.17	63	*	P166A	2.99	38	*
P54A	3.34	61	*	P66A	3.71	23	*
P145A	3.66	58	*	P131A	3.84	20	*
P133A	3.74	57	*	P104A	4.00	17	*
P43A	3.85	56	*	P20A	4.22	13	*
P123A	4.07	53	*	P68A	4.23	12	*
P194A	4.37	50	*	P63A	4.25	12	*
P32A	4.45	49	*	P128A	4.31	11	*
P116A	4.68	46	*	P52A	4.35	10	
P70A	4.76	45	*	P55A	4.38	9	
P60A	4.9	43	*	P183A	4.41	9	
P109A	4.95	43	*	P7B	4.46	8	
P45A	5.46	37	*	P29A	4.55	6	
P85A	5.48	37	*	P191A	4.60	5	
P139A	5.54	36	*	P24A	4.63	4	
P113A	5.87	22	*	P46A	4.69	3	
P174A	5.93	31	*	P95A	4.74	2	
P120A	6.25	28	*	P59A	4.75	2	
P144A	6.29	27	*	P58A	4.77	1	
P49A	6.45	26	*	P71A	4.78	1	
P97A	6.87	21	*	Control	4.83	0	
P146A	6.91	20	*	P98C	4.92	-3	
P127A	7.06	18	*	P67A	4.98	-3	
P99D	7.20	17	*	P94A	4.98	-3	
P3A	7.23	16	*	P87A	5.10	-6	
P159A	7.25	16	*	P181A	5.12	-6	
P74A	7.26	16	*	P156A	5.15	-7	
P84A	7.34	15	*	P88A	5.31	-10	
P1A	7.46	14	*	P26A	5.33	-10	
P153A	7.48	14	*	P118A	5.43	-12	
P51B	7.73	11	*	P170A	5.47	-13	
P112A	8.11	6		P158A	5.51	-14	
P107A	8.18	6		P9D	5.51	-14	
P122A	8.22	5		P28A	5.63	-17	
Control	8.66	0		P185A	5.68	-18	
P119A	8.98	-4		P161A	5.77	-19	
P37A	9.30	-7		P149A	5.78	-20	
P10C	9.37	-8		P53A	5.95	-23	
P129A	9.44	-9		P35A	6.1	-26	
P8A	9.84	-14		P69A	7.78	-61	
P147B	10.48	-21					
P135A	11.27	-30					
P157A	11.8	-36					

a: leaf rheology expressed in N/mm.
b: % of variation compared to the non-inoculated disk.
c: * shows results significantly different from *Venturia inaequalis* only with Ttab.= 1.67, 1.68.

TABLE IV: Continued

THIRD SET				FOURTH SET			
Isolates	*Mean (a)*	*% (b)*	*T Test (c)*	*Isolates*	*Mean (a)*	*% (b)*	*T Test (c)*
P41A	2.44	35	*	P132A	1.95	60	*
P152A	2.49	34	*	P176A	2.36	28	*
P73A	2.66	29	*	P143A	2.59	21	*
P62A	2.77	26	*	P114A	2.61	20	*
P83A	2.78	26	*	P31A	2.92	11	
P42A	2.79	26	*	P175A	2.98	9	
P105A	2.87	23	*	P141A	3.01	8	
P155A	2.91	22	*	P165A	3.11	5	
P90A	3.08	18	*	P22A	3.25	1	
P140A	3.16	16	*	P164A	3.27	0	
P184A	3.17	15	*	Control	3.27	0	
P30A	3.24	14	*	P148A	3.42	-5	
P75A	3.37	10		P106A	3.49	-7	
P147A	3.39	10		P142A	3.61	-10	
P188A	3.39	10		P10A	3.69	-13	
P124A	3.42	9		P108A	3.73	-14	
P101A	3.49	7		P163A	3.79	-16	
P102B	3.49	6		P50A	3.83	-17	
P76A	3.55	5		P5A	3.89	-19	
P157A	3.59	4		P15A	3.92	-20	
P89A	3.72	1		P173A	3.96	-21	
Control	3.75	0		P79A	3.96	-21	
P154A	3.77	-1		P13B	4.02	-23	
P195A	3.80	-1		P93A	4.02	-23	
P190A	3.82	-2		P18A	4.04	-24	
P182A	3.99	-7		P171A	4.08	-25	
P57A	4.00	-7		P40A	4.11	-26	
P169A	4.02	-7		P86A	4.13	-26	
P151A	4.04	-8		P4A	4.14	-27	
P14B	4.27	-14		P111A	4.15	-27	
P117A	4.3	-15		P178A	4.21	-29	
P48A	4.32	-15		P138A	4.22	-29	
P64A	4.34	-16		P92A	4.25	-30	
P187A	4.38	-17		P103A	4.30	-31	
P115A	4.42	-18		P80A	4.31	-32	
P77A	4.49	-20		P78A	4.32	-32	
P39C	4.54	-21		P38A	4.33	-32	
P186A	4.56	-22		P134A	4.34	-33	
P100A	4.59	-22		P125A	4.42	-35	
P61B	4.75	-27		P6A	4.42	-35	
P47A	4.83	-29		P126A	4.46	-36	
P150B	4.92	-31		P172A	4.48	-37	
P160A	5.11	-36		P12A	4.51	-38	
P136A	5.12	-37		P167A	4.74	-45	
P2B	5.35	-43		P196B	4.75	-45	
P179A	5.94	-59		P137A	4.77	-46	
P192A	6.36	-70		P23A	4.80	-47	
P193A	6.63	-77		P82A	4.81	-47	
P162A	6.71	-79		P177A	4.88	-49	
P65A	7.3	-95		P27A	5.22	-60	
				P11A	5.30	-62	
				P81A	5.57	-70	

TABLE V: Effect of fungal isolates on ascospore production of *Venturia inaequalis* inoculated on apple leaf discs.

Isolates	Log.(a)	% (b)	TTest (c)	Vis. obs. (d)	Isolates	Log.(a)	% (b)	TTest (c)	Vis. obs. (d)
A150A	0.00	100.00	*	0.000	P48A	2.09	85.61		0.333
A62A	0.00	100.00	*	2.667	A160A	2.14	83.77		
A66A	0.00	100.00	*	3.000	P115A	2.37	72.32		1.333
A80A	0.00	100.00	*	2.000	A159A	2.42	68.87		1.667
A81A	0.00	100.00	*	1.333	A50A	2.43	67.94		0.000
A9A	0.00	100.00	*	1.667	P141A	2.48	64.05		3.000
P127A	0.00	100.00	*	0.333	A45A	2.55	58.15		1.000
P143A	0.00	100.00	*	4.000	P75A	2.55	57.50		0.333
P145A	0.00	100.00	*	3.667	P76A	2.56	56.59		0.333
P148A	0.00	100.00	*	2.667	A136B	2.62	50.00		1.667
P180A	0.00	100.00	*	2.667	P26A	2.62	49.99		3.000
P182A	0.00	100.00	*	4.000	P37B	2.63	49.71		3.000
P65A	0.00	100.00	*	2.333	P25A	2.64	47.64		0.000
A74A	0.46	99.78	*	1.667	P29A	2.66	46.25		0.667
A74B	0.46	99.78	*	2.000	P112A	2.67	44.97		3.000
P136B	0.46	99.78	*	2.000	P1A	2.68	43.18		0.667
A54A	0.50	99.74	*	2.000	P150A	2.70	40.49		1.000
P183A	0.53	99.71	*	3.333	P35A	2.75	33.37		2.667
P32A	0.61	99.64	*	3.333	A83A	2.76	31.88		0.000
P132A	0.71	99.51	*	4.000	P18A	2.77	30.71		2.667
P152A	0.71	99.51	*	4.000	P30A	2.78	28.23		0.000
P117A	0.99	98.94	*	3.000	P186A	2.81	23.74		1.000
P28A	0.99	98.94	*	1.333	P71A	2.81	22.52		0.000
A49A	1.07	98.72	*	1.000	P59A	2.81	22.44		1.333
P122A	1.07	98.72	*	1.333	P53A	2.82	22.28		3.000
P149A	1.08	98.68	*	3.000	P184A	2.82	22.20		0.000
A82A	1.11	98.57	*	2.000	A162A	2.82	21.24		1.000
A43A	1.49	96.46	*	2.000	P66A	2.83	20.55		2.333
P158A	1.50	96.32	*	0.000	A69A	2.85	15.24		2.667
P131A	1.57	95.67	*	3.000	A79A	2.90	5.03		0.000
P166A	1.57	95.67	*	2.333	P151A	2.91	3.02		1.667
A55A	1.74	93.64	*	2.000	P107A	2.92	0.66		1.000
A148A	1.77	93.17	*	2.000	Control	2.93	0.00		2.000
A111A	1.89	90.97	*	3.000	P69A	2.94	-3.78		0.667
A72A	1.89	90.91	*	0.333	P114A	2.94	-4.31		1.000
P185A	1.90	90.73	*	2.667	A95A	2.96	-7.50		0.000
P157A	1.94	89.75	*	0.000	P155A	2.96	-8.69		3.000
P135A	1.96	89.31	*	1.667	P133A	2.97	-9.73		4.000
P140A	1.96	89.27	*	2.667	P124A	2.97	-11.85		0.333
P179A	2.02	87.64	*	0.333	A57B	2.99	-16.56		3.000

a: Log (x+1) of ascospore production of *Venturia inaequalis* per cm^2.
b: % of reduction in ascospore production compared with disc with *Venturia inaequalis* only.
c: * shows statistically different from the control (P=0.05) and LSD=0.8566.
d: Mean of the visual observation of the suspension resulting from the leaf discs bubbled during the ascospore extraction. 0: no decomposition, 1: fairly decomposed, 2: decomposed, 3: very decomposed, 4: completely decomposed

TABLE V: Continued

Isolates	*Log.(a)*	*% (b)*	*TTest (c)*	*Vis. obs. (d)*
A107A	3.00	-19.17		3.000
P49A	3.00	-19.42		1.000
P68A	3.01	-21.19		1.500
A151A	3.02	-23.44		2.000
P62A	3.02	-23.98		2.333
P118A	3.04	-31.38		1.333
P139A	3.08	-41.85		1.000
P156A	3.14	-64.79		0.667
P58A	3.14	-64.94		3.000
P86A	3.15	-69.66		0.000
A157A	3.17	-74.11		1.000
A112A	3.17	-77.71		3.000
P144A	3.18	-78.38		0.667
A120A	3.20	-89.28		1.333
P67A	3.21	-91.13		2.000
A125A	3.25	-110.23		3.333
A154A	3.25	-111.24		0.333
A144A	3.26	-116.57		0.000
P129A	3.26	-116.75		1.333
A156A	3.26	-117.11		3.000
A113A	3.27	-119.85		1.333
P94A	3.27	-120.60		1.667
P103A	3.27	-122.47		1.667
P73A	3.27	-123.83		3.333
P60A	3.28	-124.44		2.667
P72A	3.28	-124.79		1.667
P116A	3.29	-132.26		1.333
A153A	3.30	-136.94		2.000
A44B	3.32	-150.69		1.333
P128A	3.34	-158.34		1.000
P45A	3.34	-162.02		2.667
P109A	3.40	-197.27		1.333
A91A	3.43	-219.74		0.667
A152A	3.44	-224.01		1.667
P120A	3.44	-228.00		2.333
P74A	3.44	-228.25		1.000
A97B	3.44	-229.53		2.667
P123A	3.50	-272.70		0.667
P61B	3.55	-320.47		3.000
P113A	3.59	-367.54		2.333

FIG. 17: Ascospores, spores of antagonists and leaf debris seen with the haemacytometer during ascospores decount.

To determine which variable was mostly correlated with ascospore inhibition, a matrix composed of the correlation coefficients and associated probabilities for the different media tested, leaf rheology and ascospore production is presented (Table VI). There was a significant correlation among the different media, but no correlation was significant between the different variables and the ascospore inhibition according to Pearson correlation coefficients. This means that ascopore inhibition was not related with the fungal growth on the different media and with leaf rheology. A non-parametric Spearmann correlation test revealed that visual evaluation of leaf decomposition was not correlated to ascospore production since the observation of the bubbled leaf discs gave a correlation of r=-0.16, which was not significant at the 0.05 level.

TABLE VI: Correlation between different variables with ascospore inhibition of *Venturia inaequalis*.

	V8[a]	WA[b]	CA[c]	PA[d]	CMC[e]	MRH[f]	PRH[g]	ASC[h]
V8	1.00[i]	0.69	0.85	0.84	0.84	0.12	0.17	-0.06
	0.0[j]	0.0001	0.0001	0.0001	0.0001	0.0452	0.0034	0.2785
WA		1.00	0.80	0.79	0.77	0.19	0.28	0.09
		0.0	0.0001	0.0001	0.0001	0.0012	0.0001	0.0893
CA			1.00	0.94	0.89	0.10	0.23	-0.02
			0.0	0.0001	0.0001	0.07	0.0001	0.7439
PA				1.00	0.91	0.12	0.21	-0.02
				0.0	0.0001	0.0374	0.0003	0.7704
CMC					1.00	0.07	0.16	0.073
					0.0	0.2488	0.0047	0.1574
MRH						1.00	-0.33	0.12
						0.0	0.0001	0.0515
PRH							1.00	0.01
							0.0	0.8542
ASC								1.00
								0.0

a to e: culture media: a: V-8 juice; b:water agar; c: chitin agar; d: pectate agar; e: carboxymethylcellulose agar
f: leaf rheology measured in N/mm (mean of 3 measurements)
g: leaf rheology expressed the variation in % with the control disc
h: ascospore inhibition (express in log (x+1)) of *Venturia inaequalis* per cm^2
i: correlation value
j: significant critical P=0.05 level

To point out more specifically the relation between growth on media, leaf rheology and ascospore production, we investigated the results separately for each test.

A total of 98 fungi were tested both on media and for ascospore inhibition. This population (98 fungi) was characterized by 66% of isolates growing on at least one medium. In the sub-population of ascospore inhibitors, 58.1% were able to grown on at least one medium, thus 41.9% did not show significant

growth on any of the media. The higher percentage of the inhibitors grew on CA (19%) and on CMC alone and CA-PA-CMC (16%). Less than 6% grew on other combinations of media. The absence of differences between the two populations (main=66%, inhibitors=58.1%) verified the fact that growth on media are not correlated to ascospore production.

A total of 74 isolates were tested for both leaf rheology and ascospore inhibition. Thirty percent of the isolates decomposed the leaves while 8% of the fungi both decomposed the leaves and inhibited ascospore production in the main population. Stated another way, 30% of this population were inhibitors, and among these inhibitors, 27% also decomposed the leaves; which means that some how leaf decomposition is related to ascospore inhibition because the frequency of decomposers is more than 3 times higher.

Another way to compare leaf rheology with ascospore inhibition was to record the aspect and the color of the suspension resulting from the bubbling of the leaf discs. In the ascospore test, using the visual observation of the suspensions, 77% of the isolates did not decompose leaves (below score 3) and 23% decomposed leaves (Table V). When looking at the fungal isolates which inhibited more than 90% of ascospore production, 33% of them were not decomposers and 67% were. It is interesting to note that the frequencies between the main population and the sub-population composed of inhibitors were completely reversed. According to this latter variable, ascospore inhibition seem related to leaf decomposition observed in the corresponding suspension.

An overview of the fungi that significantly inhibited ascospore production by *V. inaequalis* and their results given by the other tests are represented in Table VII. This table may give some idea of the modes of antagonism in some fungi. The main difference in the fungi that had a significant effect on ascospore inhibition was their capacity to decompose leaves and/or grow on amended media with different substrates linked to decomposition. For example, A74B, P122A, P136B and P179A probably used antibiosis as a mode of action, since they did not grow well on the tested media, had no effect on rheology and did not decompose the leaf discs during the spore extraction. Isolates P127A, A74A, A49A, A72A, A55A, may use hyperparasitsm (growth on CA) with or without antibiosis for the same reasons. On the another hand, P143A, P145A, P183A, P117A, P32A, P140A and A111A had different indices of decomposition (CMC, rheology and aspect of the suspension). It is preferable to base our observation on the decomposition of the suspension rather than on media or on rheology. The relation between the media and the appearance of the suspension are often contrary. For example, P143A A111A and P32A had no growth on CMC but decomposed the leaves. On the other hand, P158A grew on CMC but did not decompose the leaf and had no effect on rheology.

Table VII: Summary of fungi inhibiting ascospore production.

Isolates	*Media (a)*	*Rheology (b)*	*Ascospore (c)*	*Susp.decom.(d)*
A150A	no	NT	100%, *	0
A81A	NT	NT	100%, *	1.33
A62A	CA	NT	100%, *	2.67
A66A	no	NT	100%, *	3
A80A	no	NT	100%, *	2
A9A	CA, PA, CMC	no	100%, *	1.67
P127A	CA, PA	NT	100%, *	0.33
P143A	no	no	100%, *	4
P145A	no	no	100%, *	3.67
P148A	NT	no	100%, *	2.67
P180A	NT	no	100%, *	2.67
P182A	NT	no	100%, *	4
P65A	no	*	100%, *	2.33
A74A	CA	NT	99.78%, *	1.67
A74B	PA	NT	99.78%, *	2
P136B	PA, CMC	no	99.78%, *	2
A54A	no	NT	99.74%, *	2.00
P183A	no	no	99.71%, *	3.33
P32A	no	no	99.64%, *	3.33
P132A	no	no	99.51%, *	4.00
P152A	NT	no	99.51%, *	4.00
P28A	CA, CMC	*	98.94%, *	1.33
P117A	no	*	98.94%, *	3
A49A	CA, PA, CMC	NT	98.72%, *	1
P122A	no	no	98.72%, *	1.33
P149A	no	*	98.68%, *	3.00
A43A	CMC	NT	96.46%, *	2.00
P158A	CMC	no	96.32%, *	0.00
P131A	NT	no	95.67%, *	3.00
P166A	NT	no	95.67%, *	2.33
A55A	CA	NT	93.64%, *	2.00
A148A	CA, CMC	NT	93.17%, *	2.00
A111A	no	NT	90.97%, *	3.00
A72A	CA, PA, CMC	NT	90.91%, *	0.33
P185A	CMC	NT	90.73%, *	2.67
P157A	NT	no	89.75%, *	0.00
P135A	CA	*	89.31%, *	1.67
P140A	CMC	*	89.27%, *	2.67
P179A	no	no	87.64%, *	0.33

a: significant growth on tested media. no refers to no growth on the media; CA, PA, CMC refer to the different media and NT for not tested
b: *refers to a significant result and no to no effect. NT: not tested
c: % of ascospore inhibition of the isolates
d: mean of the visual observation of leaf discs decomposition. See Table V for abbreviations

3.5 Discussion

Even though this was not the objective of the study, when compiling the results, it is clear that the method of screening antagonists using growth on amended media and leaf rheology are not adequate and are not a good indication of ascospore inhibition. However, knowledge of enzymatic activity and leaf decomposition will be useful in elucidating the mode of action of potential antagonists to *V. inaequalis*.

Growth on carboxymethylcellulose (CMC) might not be the best representative source of the cellulose present in leaf tissue, and the nutritional needs of the fungi are probably different and more complex than what is present in the media. Although the growth on the tested media was significantly higher than on V-8 for several fungi, a part of the growth may be due to the fungal reserves or to impurities in the agar, as many grew on water agar alone. It is interesting to note that from the fungi previously isolated from apple leaf litter, 66% had a significant growth on CMC and from the ones having a significant growth on any of the media, the ones growing on CMC represented 82% of the fungi. Althought tests on media are not correlated with ascospore inhibition, the percentage (19%) of inhibitors growing on CA only, means that 1/5 of the inhibitors might not be detectable with rheology since rheology is based on decomposition of leaf components, which lack chitin. This portion of fungi may be interesting in parasitism since the possible production of chitinase can degrade the chitin, a main componant of fungal cell walls.

For leaf rheology, about one third of the tested isolates had a significant effect on leaf decomposition and this proportion is maintained when compared to ascospore inhibition (27.3% of the decomposers also inhibited ascospore

production). When the total population tested is considered, including the ones having a significant effect on ascospore inhibition, the frequency of decomposers are 3.4 fold higher in the sub-population of inhibitors. This difference in the frequencies of double-acting fungi suggest that several leaf decomposers are also effective in ascospore inhibition. This also suggests that leaf decomposition may be an explanation of the mode of action of one third of the ascospore inhibitors. Because leaf rheology was tested on surface sterilized leaves, the decomposition observed may not be due only the inoculated isolate but also to inhabitants deep in the leaf. The experiment in Petri plates filled with wet silica provided a very moist environment, probably enhancing decomposition. In fact, it was noticed that controls receiving no inoculation were also more or less decomposed. This observation affects the homogeneity in the rheology of the different controls and reduces the possibility of selecting a good decomposer since the increases in minimal rheology value an isolate needs to achieve to be effective in decomposition.

The experiment on ascospore inhibition revealed that 32% of the tested fungi significantly inhibited ascospore production. Amongst these inhibitors, more than 33% also decomposed the leaf discs in a way that the ascospore suspension had to be diluted to permit spore observations. Also, 30% of the fungi that significantly inhibited the spore production had also decomposed the leaf discs. On the other hand, the dilution may have resulted in a loss of detection sensitivity by the haemacytometer. In fact, it was found that at least 2 fungi inhibited ascospore production by more than 98%, had completely decomposed the leaf, but had no effect on leaf rheology and did not grow on CMC agar. Thus, the absence of ascospores in these tubes may be due to a lack of experimental resolution. Despite this hypothesis, more than 9 of the 40

inhibitors did not decompose the leaf discs during the bubbling, some were not effective in the rheology test and some showed only growth on CA or not on any media. This suggests a mode of antagonism like antibiosis and parasitism for these fungi. Twenty percent of the isolates seemed to affect ascospore production by acting as competitors and degrading leaves.

Elucidating competition as the mode of action is more complex than studying hyperparasitism or antibiosis. Hyperparasitism is generally recognized by the observation of hyphal penetration of the host, hyphal interactions and destruction of the host tissues. Dennis and Webster (1971) observed co-culture of species of *Trichoderma* and susceptible fungi with microscopy. Among the 80 isolates of *Trichoderma* tested, 70 showed coiling around a susceptible fungus when the two fungi were in contact. Antibiosis was assessed the same way, but the observations were done before contact between hyphae of the tested fungus with *Trichoderma* hyphae. Inhibition of growth, vacuolation and coagulation of cytoplasm of the tested fungus without any contact provided evidence that *Trichoderma* produced diffusible antibiotics. For example, *Chaetomium globosum*, was shown to be effective against apple scab by producing antibiotic substances (Cullen and Andrews, 1984). Antiobiosis can also be demonstrated by the use of culture filtrates. For example, cell-free cultures from *Talaromyces flavus* killed microsclerotia of *Verticillium dahliae in vitro* and in soil (Fravel, 1988). As opposed to the last two mechanisms, demonstrating antagonism by nutrient competition has to be achieved indirectly. Showing this mode of action by dual culture is not appropriate since the two other modes can interact. When a tested fungus has an effect on the inhibition of the disease and the tested fungus shows no antibiosis or hyperparatism that competition is suggested as the mode of action. The next step is to investigate

the key compound implicated in competition. Uptake of 14-C-labeled amino acids by different phylloplane bacteria and yeasts was related with the degree of inhibition of spore germination of plant pathogens (Blakeman and Fokkema, 1982). Studying nutritional competition is complex largely because several chemical substances are present in different quantities and diversity and quantity fluctuate over time. Importance of uptake of amino acids by microorganisms in relation to biological control of *Botrytis cinerea* was studied by Blakeman and Brodie (1977). Even if positive correlations between uptake of amino acids and inhibition of germination of *Botrytis cinerea* conidia were found in vitro, the authors were unable to conclude on the role of competition in the inhibition of *Botrytis* under natural conditions. Nutritional competition has also been studied in relation to infection of beets by *Phoma betae* (Warren, 1972). He demonstrated that when pollen grains are slowly released on beet leaf surfaces, the saprophyte population maintained nutrients in low levels, so that infection by *Phoma betae* was prevented. Competition for site of infections has been demonstrated for *Condosterium purpureum* the causal agent of silver leaf of apple trees (Grosclaude, 1970). The antagonist, *Trichoderma harzianum* develops in pruning wounds and colonizes underlying wood layers and causes the formation of vascular gummosis which inhibits the development of the pathogen in the tree vascular system (Grosclaude, 1983).

Finally, there are two main approaches available to determine potential antagonism: dual culture technique and microscopical observation. These two methods give indications of antibiosis and hyperparasitism. On the other hand, we have to keep in mind that the failure of the candidates to produce zones of inhibition should not lead to rejection because nutrient competition was not assessed.

3.6 Conclusion

This chapter on leaf decomposition as a whole experiment, demonstrated that leaf decomposition alone is not a revelent parameter to screen antagonist against apple scab. No significant relation between leaf decomposition and ascospore inhibition was detected. On the other hand, we found several potential antagonists, since 40 fungal isolates reduced significantly ascospore production by more than 87%. These fungi seem to exert their antagonism in different ways, leaf decomposition being one of the several combinations of mode of antagonism. In the antagonist population, at least 30% decomposed the leaves, suggesting that competition for the substrate express as leaf decomposition may be responsible for one third of the antagonism detected among the significant inhibitors.

3.7 Literature cited

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4. GENERAL DISCUSSION AND CONCLUSION

The apple scab pathogen, *Venturia inaequalis*, is responsible for one of the major diseases on fruits. The *Venturia* life cycle is comprised of two distinct stages. The saprophytic stage occurs during the fall and the spring seasons when the fungus overwinters in infected apple leaf litter. At the end of this stage, sexual spores, ascospores, are produced and those spores, once they have germinated on new apple buds, leaves and fruits,provoke the parasitic stage. This phase is characterized by many cycles of asexual spore production (conidia) which attack other leaves and fruits.

The disease is controlled by fungicides, but with the negative environmental impacts of these products and the appearance of resistance of the pathogen population to the fungicides, other approaches of disease control have to be studied. Cultural control using different methods to enhance leaf litter decomposition (shredding, urea application) and control by host resistance are alternatives to chemical control, but both of them were shown to be not sufficient by themselves to control apple scab. A third alternative, biological control is an option which can be used in combination with other methods to control the disease. Since the begining of 1950, studies on leaf litter microflora for its potential in antagonism were conducted. The most studied and effective antagonists are *Athelia bombacina* and *Chaetomium globosum* in the 1970-1980. Despite their good results *in vitro*, *C. globosum* was found to be not as effective in the field, and *A. bombacina* may not be able to survive the winter conditions in Quebec.

Whatever the different approaches used to develop biological control, the first step is to obtain a collection of the widest number of species possible which are in contact with the pathogen to increase the chances of finding possible antagonists. In chapter two, fungi from apple leaf litter were collected in spring and in autumn in different abandoned orchards. This study demonstrated that the microflora of dead leaves is diverse and that each sampling site is different in the composition of their inhabitants. The results of this study suggest that a potential antagonist will be in the presence of several competitors, these competitors being different according to the specific site. This means that the ecology of the microflora might be an important factor in successful establishment of a biocontrol agent. Furthermore, it will also provide useful information on the kind and the complexity of the biotic environment in which the antagonist would be released. On the other hand, this collection of fungi is probably one of the largest isolated from dead apple leaves, providing increased chances of finding antagonists which interfere with the apple scab pathogen. This collection is the basis of future studies, and is a precious element in the development of biological control of this costly and destroying disease.

Another step in the development of a biocontrol agent, is to get information on the mechanism of antagonism. In this reseach we evaluated the effect of leaf degradation by different fungal isolates in relation to their effect on ascospore production by *Venturia inaequalis*. When compiling the results, the absence of correlation first suggested that screening antagonists using growth on media and with leaf rheology are not adequate and is not a good indicator of ascospore inhibition. The lack of correlation between leaf degradation and ascospore inhibition can be explained by the different mechanisms by which the

fungal isolates exert their antagonism. However the frequency of decomposers in the rheology test were 3.4 fold higher in the sub-population of ascospore inhibitors than in the main population. This also suggests that leaf decomposition may be an explanation of the mode of action of one third of the ascospore inhibitors. On the other hand, a more important result is that the experiment on ascospore inhibition revealed that 32% of the tested fungi significantly inhibited ascospore production, bringing the collection down to 40 interesting fungi and among these inhibitors, more than 33% had also decomposed the leaf discs. In summary, these inhibitors seem to exert their antagonism in different ways, leaf decomposition being one of the several combinations of modes of antagonism.

Despite complexity of relationships between antagonists and pathogens when competition is the principal mode of action, we believe that competition has advantages over the other modes of action in biological control of apple scab. Using hyperparasites implies at least two difficulties. To make hyperparasite effective, it has to be in intimate contact with the pathogen to allow hyphal interactions which may cause delivery problems. Also, the pathogen has to be present in a sufficient concentration to ensure the survival of the parasites, since parasites often feed on their host. This last point implies that it will probably be necessary to keep a level of apple scab high enough to allow the survival of the parasite but under economical level. When antagonists function through antibiosis, it may be easier to extract the toxic compound and use it directly against the pathogen. However, this toxin must be stable in a wide range of environments to avoid degradation as was the case with *Chaetomium globosum* which resulted in failure of biocontrol of apple scab. Using competition for biological control is not without difficulties. However, because of

the saprophytic stage of *V. inaequalis* which last for several weeks in a relatively stable environment, use of competition for nutrients and ecological niches is promising.

Despite the impossibility to make conclusions of the specific importance of leaf decomposition in relation biocontrol of apple scab, the fungal collection of more than 300 isolates was reduced to about 40 fungi with potential as biocontrol agents. In addition, for some of these, important knowledge on their relationship with leaves was acquired and some hypotheses on the mode of action can now be formulated for eventual tests.

This research represented an important step in elucidating the mode of action of antagonists to *Venturia inaequalis*. The understanding of the mechanisms by which biocontrol agents act is necessary to develop and to favor the exploitation of the candidate in the field. Moreover, studies on mode of action should also enable important information for future investigations on the proper timing of application, and the proper formulation of the biocontrol agent.