Genetic Diversity of *Entomosporium mespili* and its Interaction with Saskatoon Berry

BY

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ABSTRACT

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Genetic Diversity of *Entomosporium mespili* and its Interaction with Saskatoon Berry
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The saskatoon (*Amelanchier Alnifolia* Nutt.) is a woody shrub from the rose family. One of the main challenges to its culture is Entomosporium leaf and berry spot (ELBS) disease, which is caused by a hemibiothrophic fungus *Entomosporium mespili* (Sacc.). Due to the limited growth of the pathogen *in vitro*, we tested several culture media, and potato dextrose agar remained the best tested medium to grow the pathogen *in vitro*. *E. mespili* showed high variations in the morphology and sporulation of its cultures on PDA, which were not related to the geographic origin of the tested isolates. The amount of spores produced by the fungus *in vitro* was significantly high compared to its sporulation in nature. The use of liquid media such as potato broth was essential to produce enough mycelium for other experiments. The pathogen produced big spherical balls of mycelium where the nutrient and the aeration were sufficient. In absence of previous protocols to extract DNA from this pathogen, we tested several methods and determined a mixture of CTAB and SDS to be best for extraction of good quality DNA from *E. mespili* grown *in vitro*. Lyophilisation of *E. mespili'*s tissue and the addition of proteinase K to the extraction buffer reduced DNA degradation. To avoid the co-precipitation of polysaccharides with DNA, we used a high concentration of salt to precipitate polysaccharides, followed by a separation by phenol:chloroform:isoamyl (25:24:1). Molecular markers including Random Amplified Polymorphic DNA (RAPD) and Amplified Fragments Length Polymorphism (AFLP) revealed a high level of polymorphism in *E. mespili*. The artificial inoculation gave different results from natural infection, with a high level of diversity both on detached leaves and seedlings. According to our tests, growing *E. mespili in vitro* reduced its pathogenicity.

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INTRODUCTION

The saskatoon (*Amelanchier alnifolia* Nutt.*)* is a perennial woody fruit-bearing shrub, native to the North American continent. It belongs to the amygdaloideae subfamily of the rose family. It is also known by the name of "small apple" due to its similarities with apple (St-Pierre, 1999). This plant was important for natives and first settlers as they used the fruits as a staple food, the wood for arrows and other tools, and the leaves for medicinal purposes. The Saskatoon shrub is mainly cultivated in the Canadian prairies due to its high yield and resistance to low temperatures and drought. Thus, its cultivation can contribute to the diversification of agriculture and the development of the fruit industry in the Prairies (St-Pierre, 1999).

The saskatoon berry industry had a growth potential comparable to that of blueberries between 2001 and 2006. The cultivated area of Saskatoon increased by 9.7%, compared to 16.7% for blueberry (Statistics Canada 2006). The fruits have an important nutritional value with 0.96 milligrams of Iron, 3.55 milligrams of Vitamin C, 162 milligrams of Potassium, and 6 grams of fibers for each 100 grams of saskatoon berries. Besides, these fruits are an excellent source of antioxidants and anticancer compounds. The berries can be consumed fresh, as well as in bakery of goods, dairy products, jams, and jellies (Mazza, 2005).

To follow the steady expansion of saskatoon berries, Manitoba Agriculture Food and Rural Initiatives (MAFRI) has been working on a development plan for this crop in the province as part of the Manitoba Fruit Industry Development Program. This plan is conducted with the collaboration of the Prairies East Sustainable Agriculture Initiative (PESAI), the Prairie Fruit Growers Association (PFGA), and the Faculty of Agricultural and Food Sciences of the

University of Manitoba. The objective of this plan is to remove the obstacles that limit the development of saskatoons in Western Canada.

The production of berries is affected by several diseases and pests, such as fire blight and saskatoon-Juniper Rust. However, *Entomosporium* leaf and berry spot (ELBS), caused by the fungus *Entomosporium mespili* remains the most serious disease affecting saskatoons on the Prairies. The disease causes a loss of $5-8 Million every year to saskatoon fruit growers. *E. mespili* is an imperfect fungus from the Ascomycotina. Its reproduction is asexual, characterized by the production of a large number of conidia conserved in specific structures called acervuli. ELBS affects 50 species of the Pomoidae subfamily. On the Canadian Prairies, saskatoons are the main hosts of this pathogen (Lange and Bains, 1994).

Currently, there is very little information about the pathogen or its host. The data available focuses only on ELBS epidemiology and its chemical control, some reports are also available on host resistance to this disease.

Lange *et al*. (1998) had conducted research to compare fungicides for their efficacy in controlling the ELBS. They concluded that chlorothalonil and propiconazole give the best results in controlling the disease. In Manitoba, only Topas 250E (propiconazol), Funginex (triforine), and Kumulus (sulfur) are registered to use for ELBS management.

Ronald et al. (2001) had evaluated the resistance of different cultivars of Saskatoon to *E. mespili*, concluding that cultivars such as Regent, Success, and Parkhill are resistant, in comparison with Smoky, Martin, and Honeywood, which are more widespread on the Canadian Prairies.

Holtslag et al. (2004) had developed a dynamic disease forecasting model in order to minimize the use of fungicides to control ELBS. This model is based on weather conditions, inoculum released, and the susceptibility of cultivars. However, more information is still needed about the local populations of the pathogen and its interaction with saskatoons to thoroughly understand the saskatoon-*E. mespili* system. Understanding this system remains a challenge due to the biotrophic mode of life of *E. mespili*. The fungus does not produce enough conidia on artificial media, and stops growing after a few transfers to new culture media. Therefore, we first need to improve the culturing of this pathogen on artificial media, before studying its genetic diversity and its interaction with the host.

The objectives of this study were to: (i) optimize culture conditions for *E. mespili in vitro*; (ii) optimize DNA extraction methods for this fungus and investigate its genetic diversity using Random Amplified Polymorphic DNA (RAPD) and Amplified Fragments Length Polymorphism (AFLP); (iii) develop an efficient inoculation method of *E. mespili* on saskatoons under controlled conditions and use it to evaluate the pathogenicity of isolates from different orchards and provinces. Ultimately, these studies will provide the first characterization of *E. mespili* populations in Manitoba and, where possible, western Canada.

1. LITERATURE REVIEW

1.1 Saskatoon berry

1.1.1 Introduction

The word saskatoon is a derivation from the cree inanimate noun **misâskwatômina.** In 1818, Thomas Nuttall identified the saskatoon as *Aronia alnifolia*. In 1825, Sprengel used the name *Pyrus alnifolia*. Later in 1834, Nuttall adopted the name of *Amelanchier alnifolia* from the Gauloise word of "small apple" (Steeves and Steeves 1990). Saskatoons are native to the interior of the Prairies region of North America, Alaska, Northwest Territories, Prairies, and North-Western United States. The plant is known by different names such as serviceberry, juneberry, shadewood, sugar pear, and grape-pear (Harris 1970). Its fruits have been consumed by native people for centuries. Then, the first settlers in North America domesticated the plant and introduced several cultivars in the 20th century (St-Pierre 1997). Saskatoon berries are characterized by a blue purple color due to the high level of anthocyanins, polyphenolic compounds known for protective effect against human diseases (Ozga *et al*. 2005). The demand for saskatoon berries has been increasing since 1999 for both fresh and processed fruit products. More than 1200 ha have been planted on Canadian Prairies, of which 600 ha in Alberta. The production was estimated to 1.35 million kg of berries (Jeffs 2003).

1.1.2 Botany of saskatoons

The saskatoon (*Amelanchier alnifolia* Nutt.) is a deciduous shrub or small tree from the rosaceous family, with multiple stems and a height of 0.3 to 6m (Olson and Steeves 1982). The vegetative growth begins in April-May and continues until August, when the buds are initiated and developed. Between August and September, the leaves fall and the buds begin dormancy

(St-Pieere 1997). Shoots of saskatoons grow from 10.5 to 21.4 cm per year; this growth is reduced as the plants mature (St-Pierre *et al*. 2005).

The inflorescence of saskatoon is a determinate reduced panicle with 8 to 12 flowers. The terminal flower develops 2 weeks after the initiation of the inflorescence (Steeves and Steeves 1990). The occasional branching of the basal floral unit has been observed in the axial of a foliage leaf. These branches bear 3 to 4 bracteoles with buds in the axil. The vegetative shoots transform to flower shoots after differentiation of the cataphylls. This transformation is characterized by the formation of a bract primordial with precocious axillary buds (Steeves and Steeves 1990).

The flowers of saskatoons are insect-pollinated, characterized by five white petals inserted in the hypanthium, five stigmas, and twenty anthers. The flowering process is initiated by the appearance of flower buds in summer and fall, followed by the development of flowers in May-June of the next season (Steeves *et al.* 1991). The sepals are formed sequentially and the hypanthium is initiated from the interprimordial zonal growth. The petals and the stamens appear in a spiral pattern around the floral meristem and form as the hypanthium extends. The styles and the stigmas are formed from the gynoecial primordial. In the basal zone, they are joined by the zonal growth to form the roof of the ovary. The gynoecial primordial is probably responsible for the initiation of ovulation (Steeves *et al.* 1991).

The new shoots appear in April-May. In the next season, these shoots become reproductive and bear flower buds. The production of fruits begins 45-60 days after flowering. Their maturity is completed between July and August (St-Pierre 1997), and their ripening after two weeks from the onset. The yield is estimated from 1.32 to 4.7 kg per shrub (St-Pierre *et al.* 2005).

1.1.3 Propagation of saskatoon

Saskatoon plants can be propagated in sexual or vegetative ways. The sexual propagation is used mainly to produce rootstocks. Saskatoon is self pollinated; the production of fruits and seeds does not require genetically distinct parents and cross-pollination (St-Pierre 1997). Harris (1961) determined that softwood cutting is the practical method of propagation for Saskatoon, due to the good rooting capability and good yield obtained.

Since 1987, Micropropagation has been used for a mass propagation of saskatoons. Murashige and Skoog medium (MS) was the optimum medium used for shoot proliferation. The addition of auxin stimulates rooting of the shoots (Pruski *et al.* 1990).

1.1.4 Saskatoon cultivars

St-Pierre (1997) has reported the presence of 26 named cultivars of saskatoon in Canada. They were selected based on yield, fruit size, color of flowers, taste, and flowering time. Smoky was one of the first cultivars released and the most dominant in orchards. The rest of the cultivars such as Honeywood, Parkhill, Nelson and others have not been largely planted due to the lack of information on performance.

1.1.5 Fertilisation

At a depth of 0 to 15cm of soil, the minimum level of macronutrients per hectare recommended in the prairies for fruit crops is 28 to 56 kg of nitrogen, 56 to 112 kg of phosphorus, and 336 to 672 kg of potassium. At 15 to 30 cm of depth, the plant requires more macronutrients, 56 to 84 kg of nitrogen, 90 to 180 kg of phosphorus, and 560 to 1120 kg of potassium (St-Pierre 1997).

The pH of soil influences the availability of the macronutrients. The optimum level of pH for saskatoons is between 6 and 7. Below pH 6, the plant shows a deficiency of nitrogen, phosphorus, potassium, sulfur, calcium and Magnesium. At pH above 7.5, the addition of micronutrients (iron, manganese, boron, copper, and Zinc) is required (St-Pierre 1997).

1.1.6 Irrigation

As a native shrub, Saskatoon was able to survive under the normal rain-fed conditions of the Prairies. However, additional irrigation improves the establishment of new plantations and increases buds development, flowering, and fruit yield (St-Pierre 1997).

1.1.7 Diseases in saskatoons

Saskatoons are susceptible to several diseases. ELBS and Berry spot remains the most devastating disease that can cause the loss of the total production of berries (St-Pierre 1997).

Saskatoon-juniper rust is caused by the rust fungus *Gymnosporangium nelsonii* (Arthur.). The disease appears as yellowish spots and swellings on both leaves and berries (St-Pierre 1997).

Canker fungus *Cytospora leucostoma* (Sacc.) infects buds and shoots. It causes drying and shriveling of buds and leaves. The bark has vertical splits and folds (St-Pierre 1997).

Powdery mildew is caused by *Podosphaera clandestine* (Lév.) The disease is characterized by the white powdery appearance on one or both leaf surfaces, with lower leaves being more susceptible to this disease (St-Pierre 1997).

Fireblight causes the wilting of new shoots and blossoms during flowering. Infected fruits are watery or oily. Fireblight is mainly spread by bees. The causal organism of this disease is *Erwinia amylovora* (Burrill.) (St-Pierre 1997).

1.2 Entomosporium Leaf and berry spot

1.2.1 History

Entomosporium diseases were first reported in Europe in the first half of the nineteenth century as common diseases on the rosaceae family. Later, they were described in the United States as leaf brownness on pear and quince (Arthur, 1885). In 1888, Entomosporium infection was detected on Hawthorns in Wisconsin (Stowell and Backus, 1966). In 1935, this disease was considered as the most destructive disease of quince in New York State. Several years later, the diseases were detected in different locations of the world including Canada, Argentina, Brazil, Japan, New Zealand, Australia, India, Israel, and South Africa. They were known as leaf blight diseases (Piehl and Hildebrand, 1936). In 1957, Entomosporium leaf spot was reported for the first time on photinia in Louisiana, before its spread to Florida and Virginia (Plakidas, 1957).

According to Stowell and Backus (1966), four species of *Entomosporium* were identified on different host plants. *E. brachiatum* Lev.,*E. maculatum Lev., E. mespili (DC.)* Sacc, *and E. thuemenii (Cooke)* Sacc. These morphologically indistinguishable species are described according to the host plant where they were identified (Sultton, 1980).

1.2.2 Causal agent

1.2.2.1 The imperfect stage

Entomosporium mespili is an asexual state fungus, from the ascomycetes group that is a specific pathogen of members of the rosaceae. It is a hemibiotroph fungus that is parasitic in living cells for some time and saprophytic on dead cells. and produces insect-like cruciform conidia, composed of 4 to 6 cells, separated by a septum and each containing a single nucleus (Sutton, 1980).

The upper and lateral cells are characterized by a conspicuous seta. The development of these conidia begins by the formation of a single cell, which is later divided to form the two-celled structure. The two lateral cells are formed from the upper part of the lower cell. The setae are elongated from the apex of the upper cell and from the tips of lateral cells. The size of conidia varies from 12 to 21µm in length and from 8 to 14µm in width. Conidia are able to germinate from each cell; however, the central cells produce more hyphae than the laterals (Stowell and Backus, 1966).

The pathogen invades host cells by septate hyphae. Later, these hyphae form a subcutaneous layer of sporogenous cells, forming the conidia. Haustoria are produced in living host cells. Each one is composed by two distinctive parts, the long slender neck with a single septum and the enlarged distal body with a single nucleus (Mims *et al*, 2000a).

E. mespili is characterized by the development of a cushion-like aggregation of hyphae, called acervuli. These small asexual fruiting bodies consist of a mass of branched hyphae, formed beneath the cuticle. They are developed on the upper surface of leaves as dark brown to black

rounded raised areas in the centers of the lesions. They become grey-white when the conidia are formed and accumulated (Stowell and Backus, 1966).

The conidiogenous cells form conidiophores. Each single conidiogenous cell ascends three to four conidia. The first conidium formed is holoblastic. The ones developed later are enteroblastic, annellidic fashion. The conidiophores erupt through the epidermis to the surface of leaves (Mims *et al*, 2000b).

1.2.2.2 The perfect stage

The perfect stage of *E. mespili* is reported as *Fabraea maculate* (Lév.). This sexual form is a discomycetous fungus, found for the first time on overwintered quince leaves in New York by Atkinson in 1897. Klebahn (1914, 1918) observed apothecia on overwintered pear leaves that were infected by Leaf spot. He named the pathogen *Entomopezia soraueri* (Kleb). He obtained the conidia of the *Entomosporium mespili* from asci in pure cultures. In 1942, Greene identified ascocarps of *Fabraea maculata* on overwintered leaves of *Crataegus oxyacantha* (Linn.) in Madison, Wisconsin, where conidia were observed in the previous fall.

The initiation of the perfect stage of *E. mespili* known also by *Diplocarpon maculatum* (Atk.) is observed in the beginning of the fall, characterized by the production of small bacilliform spermatia in a subcuticle, the appearance of spermagonia, which is an acervulus-like structure formed on the lower surface of leaves, and the development of a stromatic cushion in the spongy parenchyma of leaves. Each cushion contains archicarps. The acervuli produce both conidia and spermatia when the fungus is changing to its sexual form. The spermatophores were found in the subcuticular layer of the leaf. Each one is formed by a short thick basal cell that bears one or

more longer cells. The mycelium is developed abundantly in the necrotic mesophyll tissues. The hyphae are coarse and composed of short cells. The apothecia are developed and attain maturity in spring, where asci are found. Each ascus contains eight hyaline bi-cellular ascospores (Stowell, and Backus, 1967).

1.2.3 Symptoms

On hawthorn, leaf spot symptoms appear as brown irregular lesions on the upper surface of the leaves. The size of each lesion is around 1-4 mm in diameter. Later, the fungus produces subcuticular acervuli (Stowell and Backus, 1966).

On Indian hawthorn, the symptoms begin with round and red spots of 1-2mm in diameter on both sides of the leaf. These spots elongate to 5mm, and form necrotic areas. The perimeter remains red and surrounded by a yellow halo, while the center turns to reddish, brown to black, and angular to irregular in outline. The lesions on the lower surface have more brownish red coloration in the center than in the upper side. Acervuli are formed in the center of spots (Schubert and Brown, 1987).

On photinia, Baudoin (1986a) observed that 91% of the lesions emerge after nine days of inoculation of detached leaves. The lesions have a form of tiny black flecks within the drop area. This period of nine days is considered as a good indicator of the final number of infection sites. After 14 days, just a few new lesions appear. In the case of severe infection, the lesions coalesce, and leaves become necrotic. The acervuli are produced subcuticularly on both surfaces of infected leaves. The disease can cause a complete defoliation and a progressive weakening of plants (Mims *et al*, 2000a).

The symptoms on Loquat (*Eriobotrya japonica* Thunb.*)* start with a tiny, discrete, reddish brown spot. Then, they appear as a purplish margin with a yellowish green halo. The acervuli are developed as ashy brown raised points in the center of the spot. The severe infections cause premature fall of the leaves, defoliation, and a drop in the vitality of the tree (Alfieri, 1969).

On saskatoon, the disease appears first as brown spots on both leaves and fruits (Van der Zwet and Stroo 1985). Later, the necrosis develops until a complete defoliation of the plant and deformation of fruits occurs (Davidson 1989).

The younger saskatoon leaves are more susceptible than the older ones (Holtslag *et al*. 2003b). Jacobs *et al*. (1996) also demonstrated that young leaves of Photinia are 10 to 30-fold more susceptible to *E. mespili* than older leaves.

1.2.4 Cycle of infection

Stowell and Backus (1966) indicated that *E. mespili* produces conidia in mid-July. The fungus penetrates into host cells on both sides of young leaves through the cuticle and cell wall, and through the stomata of the older leaves. The penetration is twice more frequent on the abaxial surface than the adaxial side. The fungus can penetrate into epidermal cells after 12 hours of inoculation at 25°C. Conidia germinate within 18 hours; they produce appressoria and penetrate through the cuticle after 48 hours. Reports showed that conidia can germinate on both sides of the leaf; however, the infection is rare on the lower surface (Baudoin, 1986a, van der Zwet and Stroo, 1985).

Piehl and Hildebrand (1936) indicated that conidia germinate and can develop germ tubes from any of the four cells. After penetration, the fungus develops a compact network of mycelium on

the epidermal layer. Leaves develop spots and exhibit chlorosis, and then they become abscised. The ultimate phase of infection appears in mid-August as defoliation of the plant (Stowell and Backus, 1966). The vegetative hyphae are produced differently according to the host plant.

On hawthorn, sparse hyphae are developed in the intercellular spaces of the mesophyll. They spread between the palisade layer and the upper epidermis, but are also present in the parenchyma (Stowell and Backus, 1966). Haustoria are produced to invade the neighboring host cells detected in the upper epidermis (van der Zwet and Stroo, 1985).

The pathogen is conserved in the infected leaves of the previous year, or in nearby infected host plants. The pathogen overwinters in fallen infected leaves in the form of mycelium. These fallen leaves are the source of innoculum for the new leaves. Splashing water is responsible for the spread of conidia (Alfieri, 1969, Lambe and Ridings, 1979).

1.2.5 Environmental conditions

The temperature and leaf-wetness duration are limiting factors that influence the infection of host plants by *E. mespili*. On saskatoons, the combination of 10 to 20°C and 6 to 24 hours of leaf-wetness are the optimal conditions for the infection to occur. However, there is no study on what happens after 24 hours. The infection decreases above and below these ranges (Holtslag *et al*, 2003b).

Baudoin (1985a) determined that the optimal temperature required for infection of photinia is around 20°C. Outside the interval 15 and 25°C, The infection declined significantly.

The leaf-wetness of 9 to 12 hours is required for the disease development. Below 6 hours of wetness, only a few lesions appear on detached leaves. The infection efficiency (percentage of number of lesions per number of conidia deposited on the surface) is 0.07% on the plant after 9 hours of wetness. This percentage increases with the duration of wetness, respectively, 0.43% at 12 hours of wetness, and 1.2% at 24 hours (Holtslag *et al*, 2003b).

The bright sun and high temperatures decrease the pathogenicity of the fungus. In darkness, *E. mespili*'s conidia are able to survive up to 12 hours of dry periods at 25°C; the pathogenicity decreases under these conditions (Baudoin, 1985a).

The initiation of the disease is triggered by the first precipitation that occurs 1 day or more after flowering. The conidia are released from acervuli after the first two hours of rainfall, which is the cause of dissemination to the canopy of the host plant. The severity of the disease on leaves is correlated with the concentration of conidia released. This correlation can be used to estimate the overwintering inoculum that will infect plants in the spring. Then, the early application of fungicides can reduce the severity of the disease (Holtslag *et al*, 2003a).

1.2.6 Host Plants

E. mespili infects several species of the rose family, quince, loquat, pear, *Pyrus* spp., saskatoons, peach, mountain ash, crabapple, *Photinia* spp., firethorn, *Amelanchier* spp., Batsch, *Sorbus sitchensis* (Roem.), *Pyracantha coccinea* (Roem.), *Prunus persica* (Linn), *Cydonia oblonga* (Mill.), *Malus* spp., and *Rahaphiolepis* spp. On loquat, the disease is known as leaf blight, caused by *E. maculatum* (Lév.) (Alfieri, 1969).

1.2.7 Growth in pure culture.

The conidia of *E. mespili* germinate and grow to a limited extent on many agar media. However, the best known development is obtained on Potato-Dextrose Agar (PDA) at 25°C, with satisfactory sporulation (Stowell and Backus, 1966). These results were similar to those obtained by Piehl and Hildebrand (1936), who found that *E. mespili* first produces white compact masses, The color of colonies are later changing to yellow and reddish brown later.

The pH of PDA media influences the growth of *E. mespili*. The optimum pH of PDA for the development of conidial cultures is between pH 6.8 and 7.4. At pH 6, the fungus grows slowly (Piehl and Hildebrand 1936).

The maximum development is obtained under temperatures of 18 to 21°C. The addition of sucrose increases the development of the fungus on PDA. Van der Zwet and Stroo (1985) indicated that the sucrose-casein medium gave more sporulation and development of the fungus.

On PDA, thiamine is required for the growth of *E. maculatum* on artificial media, and the lack of this vitamin is for the most part responsible for the poor growth on PDA media. The addition of thiamine to potato dextrose agar increases the development of *E. maculatum* by five times and the production of conidia by thirty times (Van der zwet and Stroo, 1985).

On sucrose-casein medium, the optimum pH value for the growth of *E. mespili* is 5.5. At pH 7.5, the fungus stops growing (Van der zwet and Stroo, 1985).

This nitrogen source has a synergistic effect with the thiamine (Van der zwet and Stroo, 1985). The fungus produces three times more spores in presence of the two substances together

(thiamine 1mg/litter, casein: 5g/liter). By contrast, the asparagine reduces the development of the fungus (Van der zwet and Stroo, 1985).

Germination is stimulated by the addition of glucose, while it is inhibited by ammonium nitrate and the pear leaf extracts. The best growth result is obtained by the addition of sucrose as a source of carbon. However the rate of sporulation is enhanced by using fructose. For glucose, the optimum concentration is 2.5% when the ratio C:N is around 20:1.

The darkness initiates the germination and the growth of the mycelium. The best of conidial production is reached when the conidia were germinated and grown for one week in darkness, and then transferred to luminosity at 100 lux for 17 days. On the contrary, the high light intensity (5,000 lux) affects the germination and the early growth of colonies. By combining the conditions above, the conidial production is 200 times more on Sucrose-casein medium than on PDA (Van der zwet and Stroo, 1985).

Although spore production is not affected, *E. mespili* grown *in vitro* rapidly loses its pathogenicity after three transfers, and becomes avirulent (Van der zwet and Stroo, 1985). The conidia kept in diseased leaves at 2°C remain more virulent than those on artificial media for 3 months. Besides, the increase of germination factors and growth negatively affects the virulence of the pathogen. van der Zwet and Stroo (1985) suggested that for an inoculation to be successful, inoculum must be from diseased leaves or a conidial suspension from the first generation on artificial media.

1.2.8 Control of Entomosporium leaf spot

1.2.8.1 Resistance of host plants

Ronald *et al*. (2001) evaluated disease response to the infection by *E. mespili* among several saskatoon cultivars. They concluded that no saskatoon cultivar has immunity to the pathogen. All of them showed moderate to high levels of incidence and severity on both leaves and fruits. However, the more resistant lines are characterized by the reduction of the rate of fungal sporulation, such as Regent, Parkhill and Success, while the common fruit bearing cultivars (Smoky, Northline) do not have the ability to restrict the pathogen's sporulation.

Leaf age is an important factor in this response. The young leaves are more susceptible to the infection than the older leaves. The resistant cultivars are hybrids between *Amelanchier alnifolia* and *Amelanchier stolonifera* (Ronald *et al*. 2001)

The injection of jasmonic acid and an extract from Canada milvetch reduces the disease levels on saskatoon leaves (Wolski *et al.* 2010). These inducers activate the synthesis and the accumulation of defense-related proteins such as PR-1, PR-2, PR-5, LOX, and PAL. The production of hydroxycinnamic acid and proanthocyanidin derivatives stimulates saskatoon's defense against *E. mespili*.

1.2.8.2 Chemical control

The majority of fungicides used so far reduce the severity of Entomosporium leaf spot on photinia leaves. The triforine (0.24-0.48 g a.i/L) or thiophanate-methyl + zinc + maneb (1.35ga.i/L) completely control the disease. The chlorothalonil (1.35 g a.i/L) or vinclozolin

(0.90g a.i./L) provide an excellent control. The triadimefon (0.30 g a.i./L) and propiconazole (0.28g a.i/L) control the disease, but they cause phytotoxicity of leaves (Lange *et al.*1998).

Chlorothalonil (970g/ha) and propiconazol (125g/ha) effectively control the Entomoporium leaf and berry spot on saskatoons. Increasing the number of applications reduces the severity of the disease. Bowen *et al.* (1994) reported that the disease levels are decreased as the number of applications is increased by using the chlorothalonil, benzimidazoles, myclobutanil and tebuconazole. Hagan *et al.* (1991) concluded that the interval of 1 to 2 weeks between myclobutanil sprays of Photinia effectively controlled the disease. The triforine is registered to be used on saskatoons in Canada. This fungicide provides a good control of ELBS (St-Pierre and Kaminski, 1993) (Cobb *et al*, 1985). Hagan *et al.* (1983) reported that *E. mespili* has probably developed resistance against benomyl and thiophanate-methyl, which are currently ineffective against this disease. Sulfur as a contact fungicide reduces the levels of infection on saskatoons. However, this metal causes pytotoxicity (St-Pierre, and Kaminski, 1993).

The control of ELBS on saskatoons was improved by using a dynamic disease-forecasting model developed by Holtslag *et al.* (2004). This model allows an estimation of disease pressure by analyzing data about phenotypic development of the plant, leaf-wetness duration, temperature inoculum released, and the susceptibility of cultivars.

2 MATERIAL AND METHODS

2.1 Fungal isolates

Samples of ELBS-infected leaves were collected from different regions of the Prairies (Manitoba, Saskatchewan, and Alberta) and Minnesota. The study was carried out on a total of fifty-six isolates. All samples were collected from infected saskatoon leaves (collected in 2009, 2010, and 2011) except the two isolates Em1-1-12 and Em2-1-12 received from infected photinia plants in 2012. Each sample from infected leaves was conserved in a dry paper towel sealed in plastic bag at 4°C.

The leaf samples were examined under the microscope to identify and isolate *E. mespili*. The identification of the causal agent was based on the presence of black acervuli in the center of necrotic spots and the typical insect-like form of conidia.

A modified protocol of Park *et al.* (2011) was used to isolate conidia of *E. mespili*. The acervuli were humidified with drops of distilled water, and then dissected with a needle. The conidial suspensions were manually sucked with a syringe.

The suspensions were spread with a sterilized loop onto new artificial media. After two to five days, single germinated spores were selected under a microscope and transferred to new media plates. Each plate contained five to six single pure colonies. The plates were sealed and incubated in darkness at 24°C for one week, then for 3 to 4 weeks under light. The conidial suspensions were harvested after 45 to 60 days, used for inoculation and mycelial production on liquid media. The rest of conidia were stored at -80°C.

Isolate name	Date of isolation	Location for collection of leaf samples	Cultivar of leaf collected
Em2-1-9	Summer 2009	Point UofM	Smoky
Em3-1-9	Summer 2009	Point UofM	Smoky
Em4-1-9	Summer 2009	Point UofM	Smoky
Em5-1-9	Summer 2009	Point UofM	Smoky
Em6-4-9	Summer 2009	Point UofM	N/A
Em7-1-9	Summer 2009	Point UofM	N/A
Em5-4-10	15/06/2010	Ashville MB	Honeywood
Em6-7-10	15/06/2010	Portage la Prairie MB	Honeywood
Em7-3-10	15/06/2010	Portage la Prairie MB	Martin
Em8-1-10	15/06/2010	Point UofM	Martin
Em9-3-10	15/06/2010	Point UofM	Martin
Em10-2-10	15/06/2010	Portage la Prairie MB	Pembina
Em11-2-10	15/06/2010	Portage la Prairie MB	Success
Em12-3-10	15/06/2010	Minitonas MB	Wild
Em14-6-10	06/07/2010	Carman MB	Thiessen
Em15-2-10	01/07/2010	Ashville MB	N/A
Em16-1-10	06/07/2010	Ashville MB	N/A
Em17-1-10	06/07/2010	Oakville MB	N/A
Em18-1-10	22/07/2010	Randall, MN	Honeywood
Em19-5-10	25/10/2010	Randall, MN	Smokey
Em21-3-10	26/10/2010	Portage la Prairie MB	Martin
Em22-1-10	26/10/2010	Miami MB	N/A
Em24-1-10	26/10/2010	Portage la Prairie MB	Success
Em26-2-10	29/10/2010	Portage la Prairie MB	Thiessen
Em28-1-10	29/10/2010	Portage la Prairie MB	Honeywood
Em29-3-10	29/10/2010	Portage la Prairie MB	Parkhill
Em30-2-10	29/10/2010	Portage la Prairie MB	Northline
Em31-3-10	01/11/2010	Portage la Prairie MB	Pembina
Em32-2-10	01/11/2010	Portage la Prairie MB	Smoky
Em34-3-10	12/11/2010	Stonewall MB	Northline
Em35-2-10	12/11/2010	Nevis Alberta	unknown
Em39-2-10	13/11/2010	Strathmore Alberta	Northline
Em40-4-10	13/11/2010	Strathmore Alberta	Smoky

Table 1: List of infected leaf samples collected (2009-2010).

Isolate name	Date of isolation	Location for collection of leaf samples	Cultivar of leaf collected
Em4-1-11	19/9/2011	Morden MB	Smoky
Em6-1-11	19/09/2011	Morden MB	Regent
Em10-1-11	10/01/2011	Smeaton SK	Northline
Em11-1-11	10/01/2011	Smeaton SK	Thiessen
Em13-1-11	26/09/2011	Gilber Plains MB	Thiessen
Em16-1-11	26/09/2011	Sifton MB	Martin
Em17-1-11	27/09/2011	Keeler SK	Martin
Em18-1-11	16/09/2011	CMCDC Portage la Prairie MB	Pembina
Em19-1-11	16/09/2011	CMCDC Portage la Prairie MB	Parkhill
Em23-1-11	16/09/2011	CMCDC Portage la Prairie MB	Honeywood
Em25-1-11	22/09/2011	Meadow Lake Sk	Honeywood
Em26-1-11	16/09/2011	CMCDC Portage la Prairie MB	Smoky
Em27-1-11	26/09/2011	Sifton MB	Parkhill
Em29-1-11	26/09/2011	Sifton MB	Nelson
Em33-1-11	26/09/2011	Sifton MB	N/A
Em1-1-12	08/08/2012	Langley BC	Photinia
Em2-1-12	08/08/2012	Langley BC	Photinia

Table 2: List of infected leaf samples collected (2011-2012).

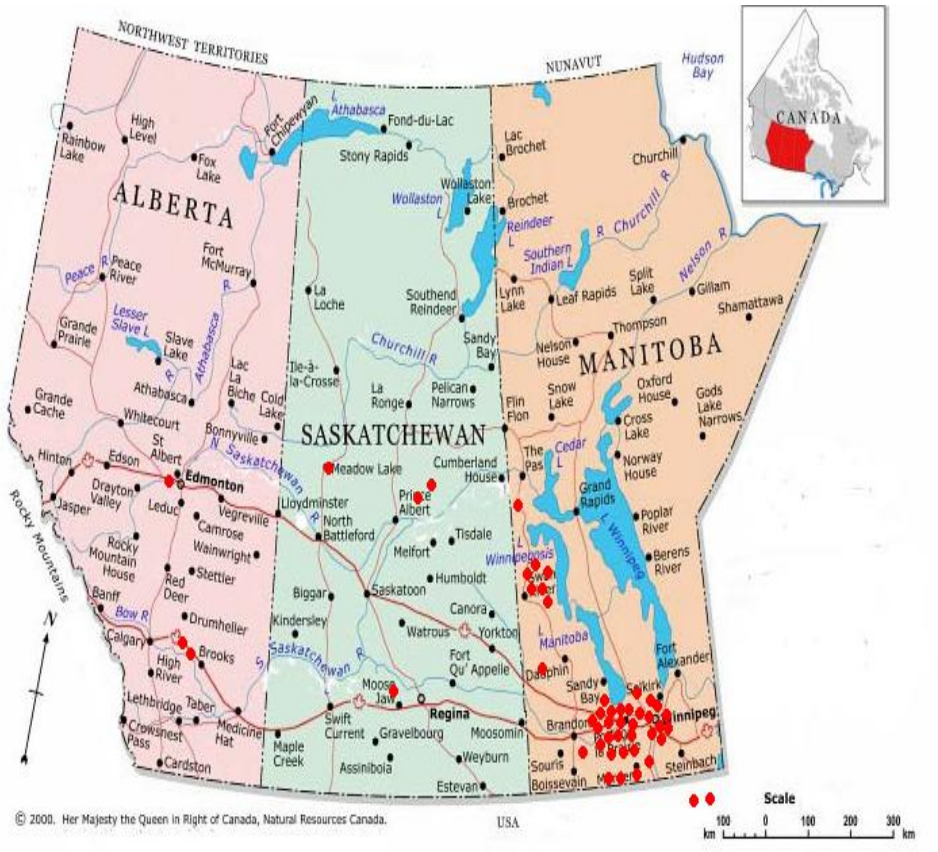

Figure 1: Location of isolates collected

2.2 Growth of *E. mespili in vitro*

Different artificial media were used to optimize the growth of *E. mespili in vitro*. The objective of this step was to obtain enough spores on petri dishes for inoculation of plants in the greenhouse, and mycelium mass in liquid media for DNA extraction. The artificial media used were, Potato dextrose Agar (PDA), Potato broth (liquid media), Corn Meal Agar (CMA), Casein-Thiamin based medium (petri dishes and liquid), and PDA-Saskatoon berry extracts medium (39g of PDA + 150ml of saskatoon juice per litter).

2.3 Morphology and sporulation of *E. mespili in vitro*

After 30 days of growth in petri dishes, single pure cultures were examined to determine morphological variation among the isolates of *E. mespili*.

The colors of single colonies were visually determined. The sizes of pure cultures were assessed by measuring the average area. We used the Image Analysis Software for Plant Disease Quantification Assess 2.1 (APS Press, Saint Paul, Minnesota. USA) for this measurement. Three replications were used per isolate.

To assess the sporulation, a conidial suspension per isolate was collected in one ml of distilled water. A single pure colony per isolate was selected in empty petri dish. One ml of distilled water was added, and then the colony was scratched gently with a loop. The conidial suspension was pipetted. The average number of spores was calculated using a hemacytometer and light microscopy. Four samples replications per isolate were measured.

The analysis of variance (ANOVA) was used to determine the difference in size colony and sporulation among isolates (SAS 9.0). Results of these analyses were represented in dendrograms to classify the isolates based on morphology and sporulation, and to determine if those parameters are related to each other and to the geography of isolates.

2.4 DNA extraction

DNA was extracted from mycelium grown both on petri dishes and in liquid media. Five protocols were used to optimize DNA extraction, since no data were available in the literature for *E. mespili*.

a- DNeasy Plant Mini Kit (Qiagen): 100 mg of mycelium from each isolate was ground with mortar and pestle. The powder was placed in 1.5 ml microcentrifuge tube containing 400µl of buffer AP1 and 4µl of RNase A. The tubes were incubated after vortexing for 10 min at 65°C. The suspensions were inverted two to three times during incubation. 130 µl of buffer AP2 was added to each suspension, followed by incubation on ice for 5 min. The lysate was centrifuged for 5 min at 14,000 rpm. The supernatant was transferred into a QIAshredder MINI spin column, and then centrifuged for 2 min at 14,000 rpm. The flow-through fraction was transferred to new tube containing 1.5 volumes of buffer AP3/E. 650 µl of the mixture was placed into a DNeasy Mini spin column, and centrifuged for 1min at 8000 rpm. The spin column was place into a new 2 ml collection tube. 500 µl of buffer Aw was added, followed by 1 min centrifuge at 6000 x g. Another 500 µl was added to the column before being centrifuged for 2 min at 20,000 x g. The spin column was place into a new 1.5 ml tube. The pellet was eluted with 50 µl of buffer AE, incubated for 5 min, and then centrifuged for 1 min at 6000x g.

b- Hexadecyltrimethyl-ammonium bromide CTAB1: DNA was extracted following a modified CTAB protocol developed by Murray and Thomson (1980). 100 mg of fresh mycelium was ground to fine powder using liquid nitrogen and a mortar and pestle. The ground tissue was transferred to 1.5 ml tube with of 500 µl prewarmed (60°C) isolation buffer (2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, 1% PVP 40,000, 0.2% β-mercaptoethanol). The mixture was incubated for 30 min at 60°C in water bath. The tube was inverted eath 10 min. An equal volume of chloform-isoamyl (24:1) was added to the tube, followed by mixing for 10 min on rotary shaker, and then centrifuging for 10 min at 5000 x g. The aqueous phase was transferred to new 1.5 ml with 0.6 volume of isopropanol. The tube was inverted several times

and then stored at -20°C for 30 min to overnight for precipitation. After 10 min of centrifuging at 14,000 rpm, the supernatant was discarded and the pellet was washed with 70% ethanol. The tube was centrifuged for 10 min at 5000 X g, the 70% ethanol was discarded, and then the pellet was dried for few minutes. The pellet was dissolved in 25 µl of TE buffer with 250µg/ml of RNase A. The tube was incubated at 37°C for 2 hours.

c- Hexadecyltrimethyl-ammonium bromide CTAB2: DNA was extracted following a modified CTAB protocol developed by Murray and Thomson (1980). 100 mg of fresh mycelium was ground to fine powder using liquid nitrogen and a mortar and pestle. The ground tissue was transferred to 1.5 ml tube with of 500 µl prewarmed (65°C) isolation buffer (2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, 1% PVP 40,000, 0.2% β-mercaptoethanol). The mixture was incubated for 20 min at 65°C in water bath. The tube was inverted eath 10 min. An equal volume of phenol:chloform-isoamyl (25:24:1) was added to the tube, followed by mixing for 10 min on rotary shaker, and then centrifuging for 10 min at 14,000 rpm. The aqueous phase was transferred to new 1.5 ml tube with equal volume of isopropanol. The tube was inverted several times and then stored at -20°C for 30 min to overnight for precipitation. The tube was centrifuged for 10 min at 14,000 rpm, then, the supernatant was discarded and the pellet washed with 70% ethanol. The tube was centrifuged for 10 min at 5000 X g, the 70% ethanol was discarded, and then the pellet was dried for five minutes. The pellet was dissolved in 500 µl of TE buffer with 10µg/ml of RNase A. The tube was incubated at 37°C for 2 hours. Equal volume of chloform-isoamyl (24:1) was added to the tube, and then mixed and centrifuged for 10 min at 5000 x g. The aqueous phase was transferred to a new 2 ml tube with 0.05 volume of 5 M NaCl and 2 volumes of 100% absolute ethanol. The tube was stored at -20°C for 30 min to

overnight for precipitation. After 10 min of centrifuging at 14,000 rpm, the supernatant was discarded and the pellet was washed with 70% ethanol. The tube was centrifuged for 10 min at 5000 X g, the 70% ethanol was discarded, and then the pellet was dried for five minutes. The pellet was dissolved in 25 µl of TE buffer.

d- Sodium dodecyl sulfate protocol SDS: DNA was extracted following Goodwin *et al.* (1992) protocol. 50 mg of ground lyophilized tissue was placed in a two ml tube with 1 ml of prewarmed (65°C) isolation buffer (025% SDS, 0.5 M NaCl, 50 mM EDTA, 100 mM Tris-HCl, pH 8.0). The mixture was vortexed and incubated for one hour at 65°C in water bath. 333 µl of 5 M potassium acetate was added, followed by incubation on ice for 20 min. The tube was centrifuged for 10 min at 14,000 rpm. The supernatant was transferred to a new two ml tube with 800 µl of cold isopropanol. The mixture was incubated for 30 min on ice. The supernatant was discarded after centrifuging for 10 min at 14,000 rpm, then; the pellet was dried for five minutes. The pellet was dissolved in 500 µl of TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). An equal volume of phenol:chloform-isoamyl (25:24:1) was added to the tube, followed by vortex and then centrifuged for five min at 14,000 rpm. The aqueous phase was transferred to a new 1.5 ml with 0.1 volume of 3 M sodium acetate and two volumes of ethanol. The tube was incubated at -20°C for two hours for precipitation. The tube was centrifuged for 10 min at 14,000 rpm, and the supernatant was discarded. The pellet was washed with 200 µl of 70% ethanol. The tube was centrifuged for 2 min at 5000 X g, the 70% ethanol was discarded, and then the pellet was dried for five minutes. The pellet was dissolved in 25 µl of TE buffer.

e- CTAB/SDS protocol: DNA was extracted following a modified SDS protocol (Dellaporta *et al.* 1983). Fifty mg of ground lyophilized tissue was ground in a two ml tube with 500 µl of

prewarmed (65°C) TES buffer (2% SDS, 10 mM EDTA, 100 mM Tris-HCl, pH 8.0, 1% PVP 40,000, 0.2% β-mercaptoethanol). 0.4 mg of proteinase k, 140 µl of 5 M NaCl, and 70 µl of 10% CTAB were added to the mixture before incubating for 20 min at 55°C in a water bath. 0.35 volume of 5 M potassium acetate was added, followed by incubation on ice for 30 min. The tube was centrifuged for 20 min at 14,000 rpm. An equal volume of phenol:chloform-isoamyl (25:24:1) was added to the tube, followed by centrifuging for 20 min at 14,000 rpm. The aqueous phase was transferred to a new 1.5 ml tube with equal volume of isopropanol, followed by incubation at -20°C for 1 hour. The tube was centrifuged for 20 min at 14,000 rpm, then, the supernatant was discarded and the pellet was dried for five minutes. The pellet was dissolved in 500 µl of TE buffer with 10µg/ml of RNase A. The tube was incubated at 37°C for 2 hours. An equal volume of chloform-isoamyl (24:1) was added to the tube, and then mixed and centrifuged for 10 min at 5000 x g. The aqueous phase was transferred to a new two ml tube with 0.1 volume of 3 M sodium acetate and 0.7 volume of isopropanol. The tube was incubated at -20°C for one hour. After 10 min of centrifuging at 14,000 rpm, the supernatant was discarded and the pellet was washed with 70% ethanol. The tube was centrifuged for 10 min at 5000 X g, the 70% ethanol was discarded, and the pellet was dried for five minutes. The pellet was dissolved in 25 µl of TE buffer.

The DNA concentration in each sample was measured using a nanodrop at 260nm. The purity was determined by calculating the ratio of absorbance at 260nm to that at 280nm.

2.5 Genetic Diversity of *E. mespili*

Two molecular techniques were used to assess the genetic diversity of *E. mespili*. Random Amplified Polymorphic DNA (RAPD) and Amplified Fragments length Polymorphism (AFLP).

2.5.1 Random Amplified Polymorphic DNA (RAPD)

For RAPD analysis, seven oligonucleotide primers were used:

OPA02: 5'-TGCCGAGCTG-3'
OPA05: 5'-AGGGGTCTTG-3'
RAPD-p6: 5'-GAAACAGCGC-3'
RAPD-p8: 5'-GGAGCCCAC-3'
RAPD-p14: 5'-GCCGTCTACG-3'
RAPD-p17: 5'-GGCATCGGCC-3'
RAPD-p21: 5'-GTGAGCGTC-3'

Each 25µl of PCR reaction contained 25ng of template DNA, 1X PCR buffer (200mM Tris-HCl pH 8.4, 500mM KCl), 3mM $MgCl_2$, 100mM of each dNTP, 0.5µM of single primer (Invitrogen Life Technoligies, Canada), and 2U of *Taq* DNA polymerase (Invitrogen Life Technoligies, Canada). The PCR reactions were performed in a DNA thermocycler (Biorad MYcycler, Applied Biosystems, CA, USA). Amplification conditions involved in initial denaturation step of 5 min at 94°C, followed by 45 cycles each consisting of denaturation step of 1min at 94°C, an annealing step of 1min at 36°C, an extension step of 2min at 72°C, and a final extension step for 15min at 72°C. The PCR products were electrophoresed on 1.2% (w/v) agarose gels in 1X TBE buffer (0.09M Tris; 0.09M Boric acid; 0.01M EDTA) containing ethidium bromide.

2.5.2. Amplified Fragments length Polymorphism (AFLP)

For the AFLP analysis, the following oligonucleotide primers were used:

EcoRI: 5'- GACTGCGTACCAATTC-3'
MseI: 5'- GATGAGTCCTGAGTAA-3'
EcoRI+ACG: 5'- GACTGCGTACCAATTCACG-3'
MseI+A: 5'- GATGAGTCCTGAGTAAA-3'
EcoRI+ACA: 5'- GACTGCGTACCAATTCACA-3'
MseI+G: 5'- GATGAGTCCTGAGTAAG-3'

And the following adapters adapters:
EcoRI.1: 5'- CTCGTAGACTGCGTACC-3'
EcoRI.2: 5'- AATTGGTACGCAGTCTAC-3'
MseI.1: 5'- GACGATGAGTCCTGAG-3'
MseI.2: 5'- TACTCAGGACTCAT-3'

AFLP reactions were carried out as described by Vos *et al*. (1995), following seven steps:

(1) Restriction Digestion: An amount of 5µl of extracted DNA (500ng) was digested in a volume of 13 µl reaction volume. Two fastdigest enzymes from Fermantas were used, EcoRI (1.25U), MseI (1.25U), and 1X fastdigest buffer. The reaction was incubated at 37°C for 2h, and then inactivated at 85°C for 5min.

(2) Ligation: This step was started by preparing adaptors. MseI adaptor was prepared by adding 50 µM of MseI.1, 50 µM of MseI.2, 10 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM EDTA. EcoRI adaptor was prepared by adding 5 µM EcoRI.1, 5 µM EcoRI.2, 10 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM EDTA. The ligation was performed by joining 7µl of adapter/ligation solution (1.25 mM EcoRI adapter, 1.25 mM MseI adapter, 1.25X ligation buffer (250mM Tris-HCl pH 7.6, 50mM MgCl2, 5mM ATP, 5 mM DTT, 25% w/v polyethylene glycol-8000), 1U T4 DNA ligase). The solution was incubated at 37°C for 2h.

(3) Pre-amplification: PCR mixtures (25µl) contained 5µl of 1:10 diluted Digestion/Ligation product, 1.2µM of Primer EcoRI, 1.2µM of Primer MseI, 1X buffer (200mM Tris-HCl (pH 8.3), 500mM KCl), 1.5mM $MgCl_2$, 100µM of each dNTPs, and 1U of Taq DNA polymerase (Invitrogen). The DNA thermocycler (Biorad MYcycler, Applied Biosystems, CA, USA) was programed for 23 cycles of 30s at 94°C, 56°C for 30s, and 72°C for 60s.

(4) Selective amplification: Four combinations of primers were used to perform the AFLP (EcoRI-ACG/MseI-A, EcoRI-ACG/MseI-G, EcoRI-ACA/MseI-A, EcoRI-ACA/MseI-G). The pre-amplification PCR products were diluted 1:50. 5µl was used as DNA template for the selective amplification. 50µl reaction volume contained 5µl template DNA, 1.2µM of Primer EcoRI-, 1.2µM of Primer MseI, 1X buffer (200mM Tris-HCl (pH 8.3), 500mM KCl), 1.5mM $MgCl_2$, 100µM of each dNTPs, and 1U of Taq DNA polymerase (Invitrogen). The reactions were performed in DNA thermocycler (Biorad MYcycler, Applied Biosystems, CA, USA) with touchdown PCR. The first cycle was set at 94°C for 30s, then 65°C for 30s and 72°C for 60s. During the touchdown phase of 13 cycles, the annealing temperature was decreased by 0.7°C each cycle. Another 23 cycles were performed at 94°C for 30s, 56°C for 30s, and 75°C for 60s. PCR products were separated on 6% acrylamide gel, and the bands were visualized by Ethidium bromide staining.

RAPD and AFLP amplified bands were scored by using the AlphaImager HP software (Protein Simple, Santa Clara, California. USA), and then analyzed as binary data (presence or absence of the band in a particular location on acrylamide gel). Polymorphic and monomorphic bands were determined for both molecular techniques. Cluster analyses were performed by NTSYS 2.1. Matrices of similarity were obtained using Simqual program. Cluster analysis was performed by the unweighted pair group method with arithmetic average (UPGMA). Results of these analyses were represented in dendrograms to classify the isolates based on genetic distance.

2.6 Pathogenicity tests

The conidia conserved at -80°C were transferred to PDA media and incubated at room tempretures for 4 weeks. The condial suspensions of *E.mespili* were collected and measured by using the hemacytometer and light microscope.

The pathogenicity tests were carried out on both detached leaves and saskatoon seedlings in order to evaluate the aggressiveness of different isolates of *E. mespili*. Negative controls treated with distilled water were used.

2.6.1 Inoculation of detached leaves

Detached leaves were collected from three different cultivars of Saskatoon (Smoky, Martin, and Northline) present in a trial field of the University of Manitoba. Three leaves per cultivar per isolate were placed on moist paper towels in petri dishes.

Three experiments of inoculation were done on detached leaves based on the availability of material.

Experiment 1: On May 11th, 2011, three isolates of *E. mespili* chosen randomly were used for inoculation of these cultivars (Em10-2-10, Em15-2-10, and Em39-2-10). The concentration of each conidial suspension was 8.10^4 spores per ml. Two different techniques were tested, using small atomizer and putting several drops of inoculum on leaves. In each experiment, a negative control of distilled water was used. The plates were incubated at room temperature for 15 days.

Experiment 2: On August 5th, 2011, three leaves per seedling of Martin cultivar previously wounded by using an abrasive powder (silicon carbide) were sprayed with conidial suspensions

of two isolates chosen randomly (Em24-1-10 and Em26-1-10). The inoculated seedlings were incubated inside a misting chamber for 48 hours, then moved to a growth chamber for 30 days.

Experiment 3: On September 2012, detached leaves from smoky cultivar were collected from an orchard in Portage la Prairie. Three leaves per isolate were placed on moist paper towels in petri dishes. New conidial suspensions were prepared from a stock of conidia kept in -80°C. The inoculation was performed by using a concentration of $1x10^5$ spores per ml. Ten drops of each conidial suspension was put on each leaf. Thirty-two isolates were used for the inoculation. The experiment was repeated three times.

2.6.2 Inoculation of saskatoon seedlings

Three experiments of inoculation were done on detached leaves based on the availability of material.

Experiment 1: On June 11th, 2011, three seedlings of Martin cultivar were sprayed with conidial suspension of $8x10^4$ spores per ml. Two isolates were chosen randomly (Em39-1-10 and Em40-1-10). Both single drops and small atomizer were used. The negative controls sprayed with distilled water were used. The plants were incubated in a mist chamber. The humidity was maximized by using a humidifier for 48 hours. Then the plants were moved to a growth chamber (18°C and 16 hours of light) for 15 days after inoculation.

Experiment 2: On November 3th, 2011, two isolates were tested on Martin cultivar, (Em4-1-11 and Em6-1-11). Both wounded and non-wounded leaves were inoculated with $8x10^4$ conidia per ml of single drops (an average of 12 drops per leaf)

Experiment 3: Ten isolates for which we were able to produce conidia were tested on the cultivar Martin seedlings during December 2011 (Em6-1-11, Em4-1-11, Em10-1-11, Em13-1-11, Em16-1-11, Em17-1-11, Em19-1-11, Em23-1-11, Em25-1-11, and Em33-1-11). Three leaves per plant per isolate were wounded and inoculated with single drops.

The disease severity was evaluated using APS Assess 2.1 software (APS Press, Saint Paul, Minnesota. USA). First, the infected leaves were scanned; then, they were threshed from the blue background of the pictures. After, the lesions were threshed from individual leaf by using hue saturation value (HSV). Finally, the Assess 2.1 software calculated the percentage of lesions per leaf.

The analysis of variance (ANOVA) was used to determine the differences of severity among isolates (SAS 9.0). Results of these analyses were represented in dendrograms to classify the isolates based of aggressiveness, and to determine if those parameters are related to each other and to the geographic distribution of isolates.

3 RESULTS

3.1 Fungal isolates

Most of the diseased leaf samples received in 2010 and 2011 were infected by *E. mespili* (Figure 2A-B). The identification of the pathogen was based on the typical form of conidia (Figure 2C-D), which consisted of four to six attached cells. The apical cells each have slender appendage. The degree of severity was variable from low to high based on the fact that some samples were treated with fungicides to control *E. mespili*.

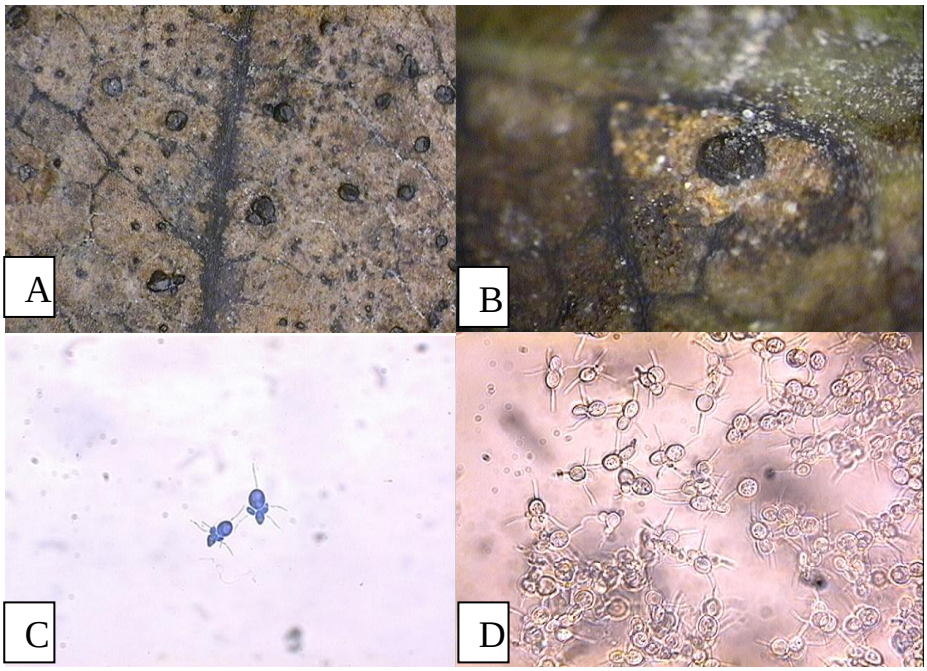

Figure 2: Acervuli and spores of *E.mespili* in leaves of Saskatoon berry.
A) Black acervuli on leaf. B) Single acervilus in the center of necrotic area.
C) Single spores. D) Conidial suspension

The examination of those infected leaves under microscope revealed the presence of black acervuli in the center of necrotic area (Figure 2A-B). Each acervulus contained a large number

of conidia. The isolation of the pathogen from infected leaves was difficult in most samples due to the presence of other pathogens in the samples; mainly *Botrytis* and bacteria.

3.2 In vitro growth of *E. mespili*

3.2.1 Solid media

E. mespili grows extremely slow on the artificial media tested. The fungus required at least 60 days to complete its asexual life cycle from germinated spore to sporulation.

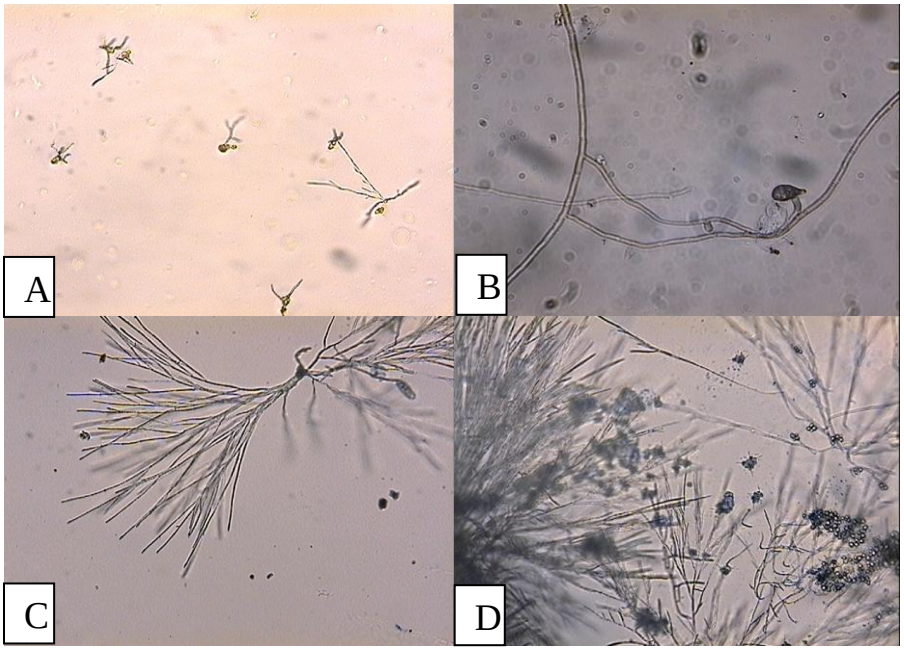

Figure 3: Growth of *E.mespili* on PDA media.
A) Germinated conidia. B) Production of septated hyphae.
C) Spread of hyphae on PDA plate. D) Development of mycelium

On PDA, spores of *E. mespili* germinate after 2 to 5 days depending on isolates (Figure 3A). Each spore produces filamentous septate hyphae (Figure 3B). The hyphae spread for few millimeters on the surface of PDA and develop conidium initials (Figure 3C). These sporogenous

cells form conidia. Hyphae and conidia are surrounded by acervuli (Figure 3A-B) (Mims et *al*, 2000).

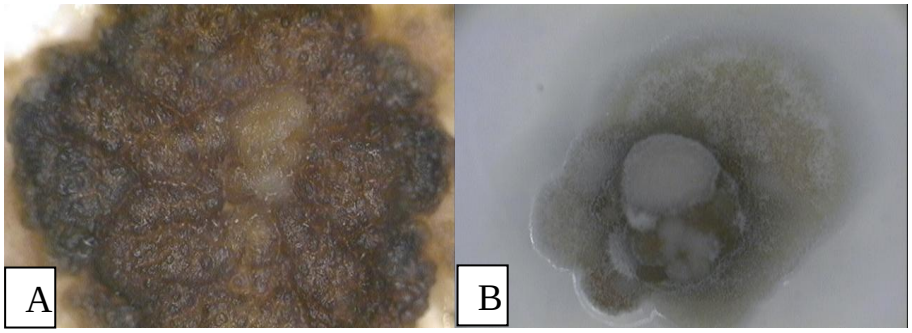

Figure 4: Growth of *E.mespili* on PDA and casein-sucrose-thiamin media.

A) Single colony on PDA. B) Single colony on casein-sucrose-thiamin media.

After one week, the mycelium is developed to a raised opaque compact mass. The different isolates produced with different morphologies. Variations in the morphology of colonies were very important, including growth rate, color, and sporulation (Figure 4A).

On sucrose-casein-thiamine based medium, the growth was higher. The white mycelium much more developed, and the color of pure culture darker than on PDA. However, the fungus did not produce conidia on sucrose-casein-thiamine-based medium as shown by the absence of acervuli (Figure 4B).

The addition of Saskatoon juice to PDA did not improve neither the growth nor sporulation of *E. mespili*. On CMA media and water agar, the conidia were able only to germinate and produce the first hyphae. The pathogen did not grow on such media.

3.2.2 Liquid media

The objective of using liquid media was to obtain enough mycelium of *E. mespili* to be used for DNA extraction. The pathogen grew well on both potato broth and casein-sucrose-thiamin media and produced spherical balls of mycelium. The growth rate was higher on casein-sucrose-thiamin media.

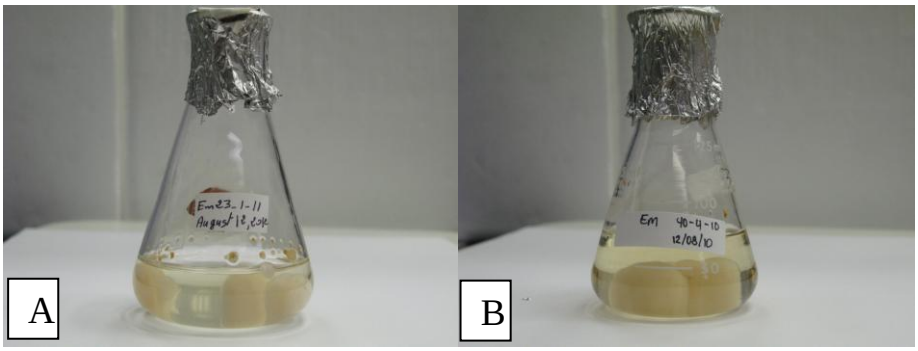

Figure 5: Growth of *E. mespili* in Potato broth medium.
A) Isolates Em231-11. B) Isolates Em40-4-10.

Like on petri dishes, the pathogen showed a significant variation between isolates regarding the growth rate and the color of the mycelium. Both potato dextrose and casein-sucrose-thiamin media (on plate and liquid) are optimal media for growing *E. mespili* in vitro (Figure 5A-B). Nevertheless, PDA was the ideal medium for sporulation.

3.3 Morphology and sporulation of isolates

3.3.1 Color of colonies

Yellowlish	Yellow & White	Brown & white	Dark Brown
Em5-4-10	Em 2-1-9	Em3-1-9	Em6-1-9
Em6-2-10	Em5-1-9	Em4-1-9	Em7-1-9
Em9-3-10	Em7-3-10	Em16-1-10	Em10-2-10
Em19-5-10	Em8-1-10	Em17-1-10	Em11-2-10
Em22-1-10	Em12-2-10	Em18-1-10	Em20-1-10
Em32-2-10	Em13-2-10	Em28-1-10	Em24-1-10
Em39-2-10	Em14-6-10	Em29-3-10	Em26-2-10
Em6-1-11	Em15-2-10		Em31-3-10
Em10-1-11	Em21-3-10		
Em13-1-11	Em23-6-10		
Em17-1-11	Em30-1-10		
Em19-1-11	Em33-2-10		
Em25-1-11	Em34-3-10		
Em33-1-11	Em35-2-10		
Em16-1-11	Em40-4-10		
Em18-1-11	Em12-1-11		
Em23-1-11	Em14-1-11		
	Em26-1-11		
	Em29-1-11		
	Em1-2-12		
	Em2-1-12		
	Em27-1-10		
	Em24-1-11		
	Em4-1-11		

Table 3: Colors of different isolates of *E. mespili.*

Two colors of colonies were observed on PDA plates, yellowish and dark brown. Among the 56 isolates examined, 41 isolates had yellowish colonies, and the other 15 isolates had brown to dark color (Table 2).

Another morphological variability was the development of aerial hyphae on the top of the raised opaque masse of the colony. This phenomenon was shown on 31 isolates, 24 isolates among the yellowish group (isolates with the same color) and 7 isolates within the dark brown group (Figure 6).

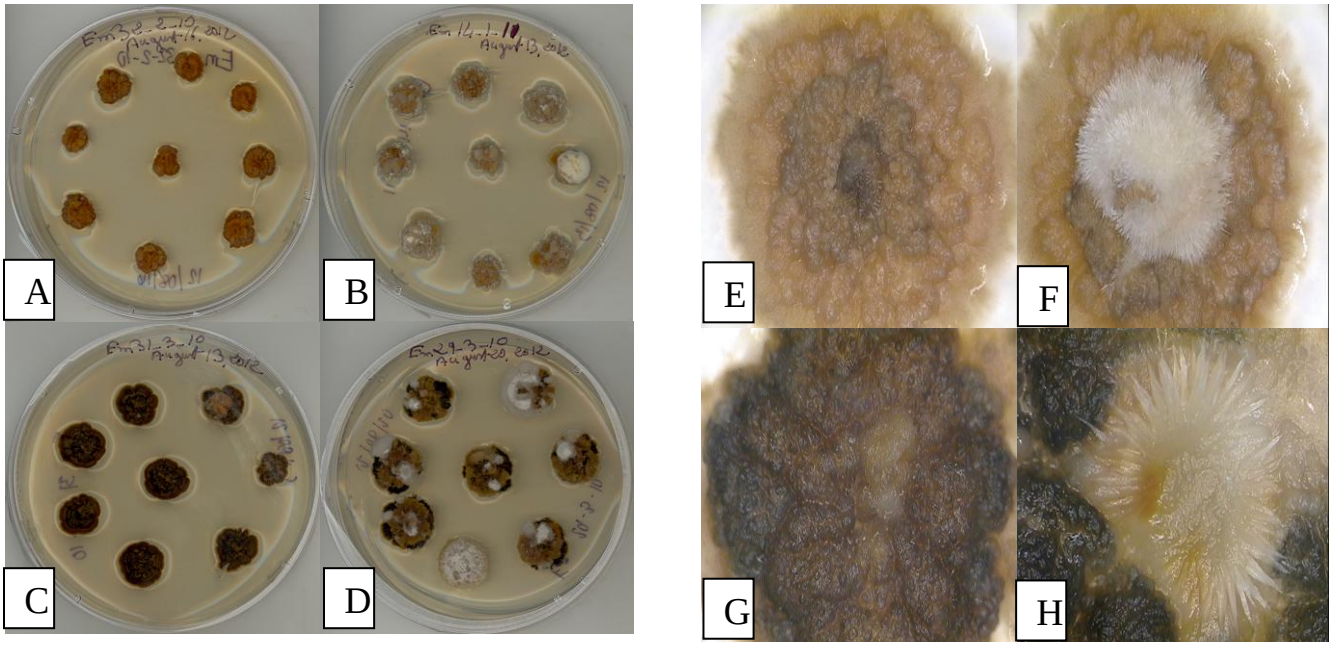

Figure 6: Color variation of *E. mespili*.
A) Yellow colonies. B) Yellow colonies with white mycelium.
C) Dark brown colonies. D) Dark brown colonies with white mycelium.
E) Single yellow colony. F) Single yellow colony with white mycelium.
G) Single Dark brown colonies. H) Single Dark brown colony with white mycelium.

The color of colonies was neither related to the geographic distribution of isolates, nor to cultivars of saskatoon berry from which they originated. However, all isolates of 2011 showed yellow color.

3.3.2 Size of colonies

The analysis of size (area) of *E. mespili* on PDA have revealed a highly variation among the 56 isolates (Figure 7). The analysis of variance (ANOVA) showed a significant variation of diameter of colonies between isolates (Number of observation= 168, F value= 10.24, DF= 55, P= 0.0001).

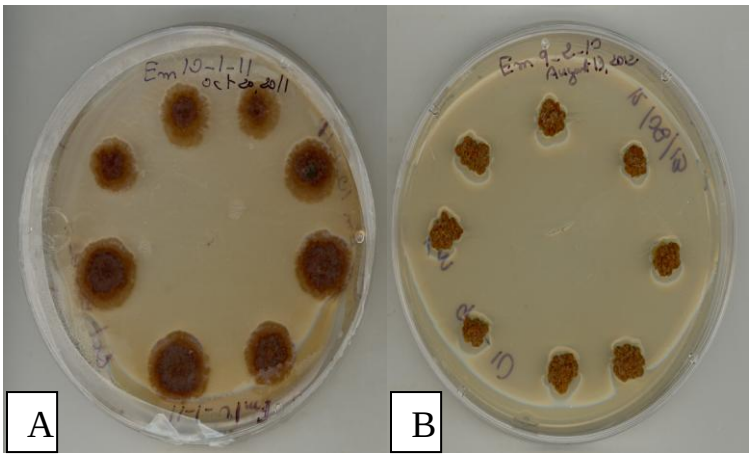

Figure 7: Size variation of *E. mespili.*
A) Pure cultures with maximal growth rate
B) Pure cultures with minimum growth rate

Student-Newman-Keuls Test determined different groups of isolates depending on the size of each individual colony. Cluster analysis was used to determine the relationship between isolate based on the growth rate.

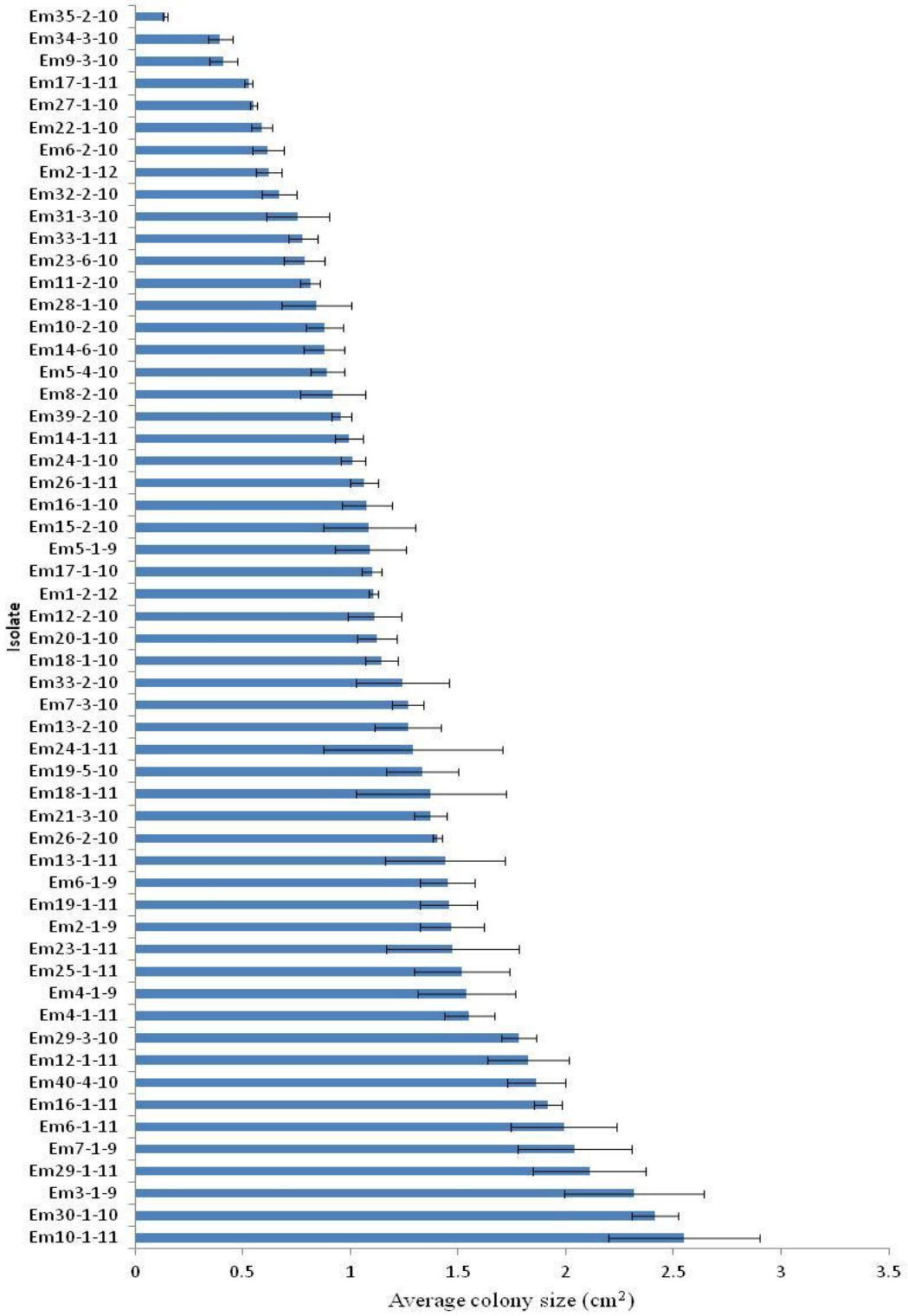

Figure 8: Average colony size in cm^2 of different isolates of *E. mespili*.

The maximum mycelial growth of *E. mespli* after 1 month on PDA was 2.55 cm^2 (isolate Em10-1-11), and the minimum size was 0.14cm^2 (Em35-2-10) (Figure 8). Cluster analysis has revealed two major distinct groups. Group A, contained 10 isolates (Em10-1-11, Em30-1-10, Em3-1-9, Em16-1-11, Em29-1-11, Em12-1-11, Em40-4-10, Em29-1-11, Em6-1-11, Em7-1-9), with growth rate above 1.78 cm^2, and Group B, had the rest of the 56 isolates tested. The growth rate was between 0.14 cm^2 and 1.55cm^2 (Figure 9).

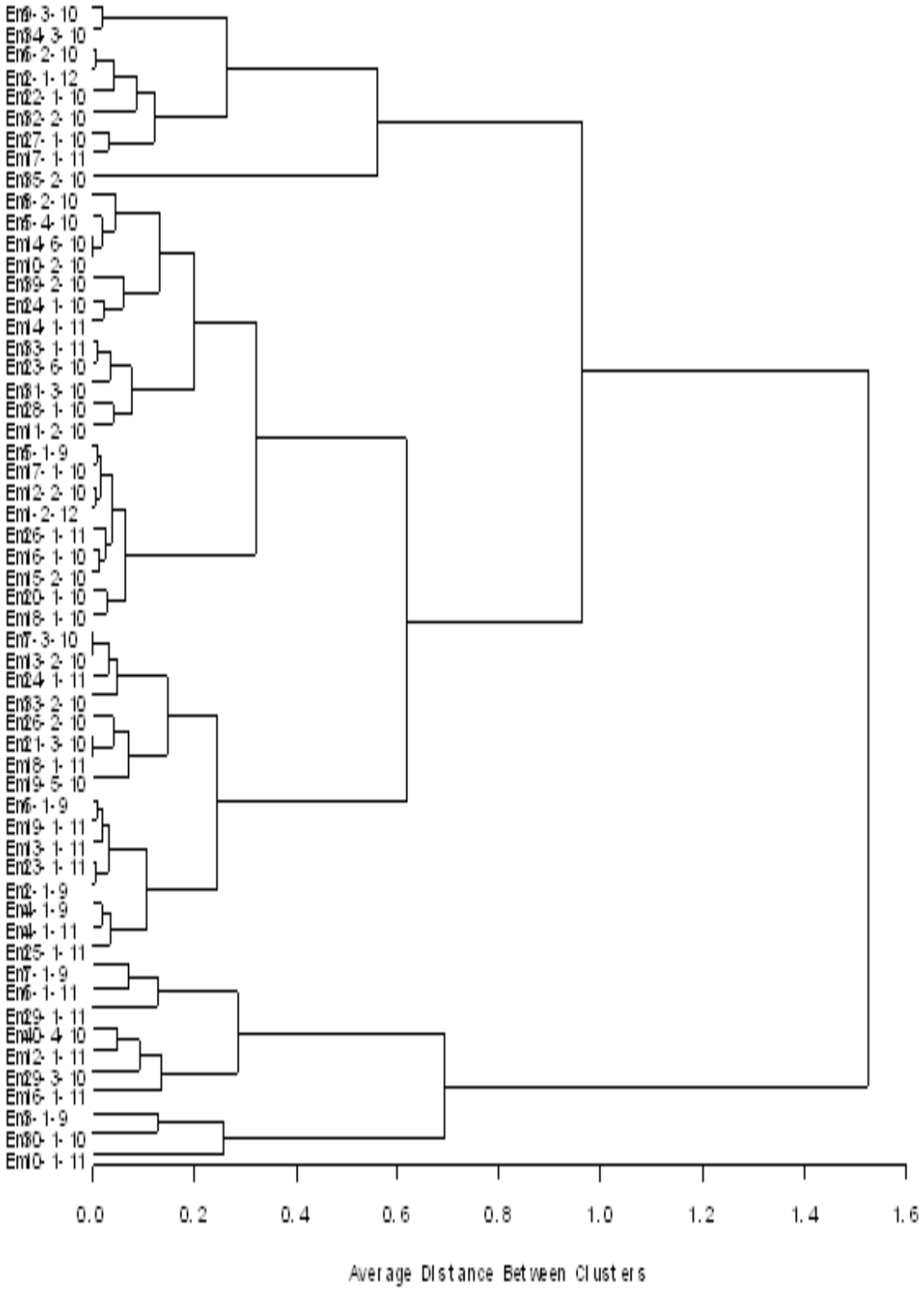

Figure 9: Cluster analysis of colony size of colonies of the tested isolates.

3.3.3 Sporulation

On PDA, the sporulation occurred at the expense of mycelial growth. Spore production of *E. mespili* was characterized by an abundant number of white acervuli in the centre of the pure culture growth (Figure 10).

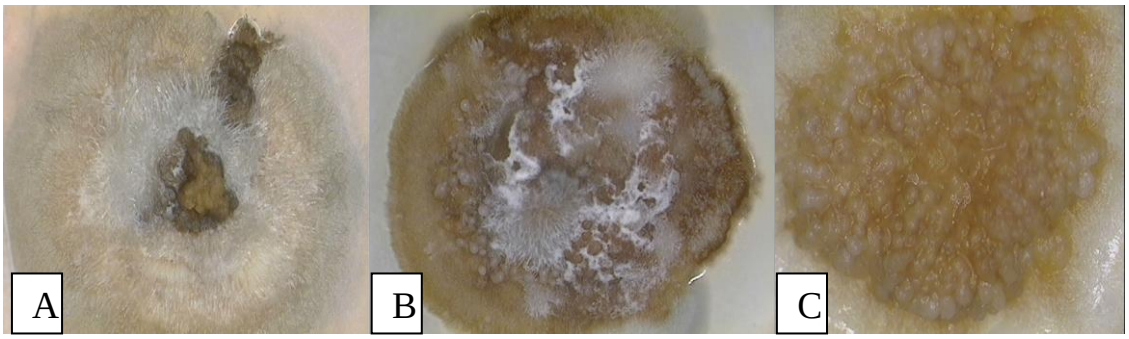

Figure 10: Sporulation of pure culture of *E.mespili.*
A) Single pure culture without sporulation
B) Single pure culture with medium level of sporulation
C) Single pure culture with high level of sporulation

The degree of sporulation was different among isolates. The maximum number of spores per single culture was $4.9x10^6$ (Figure 11).

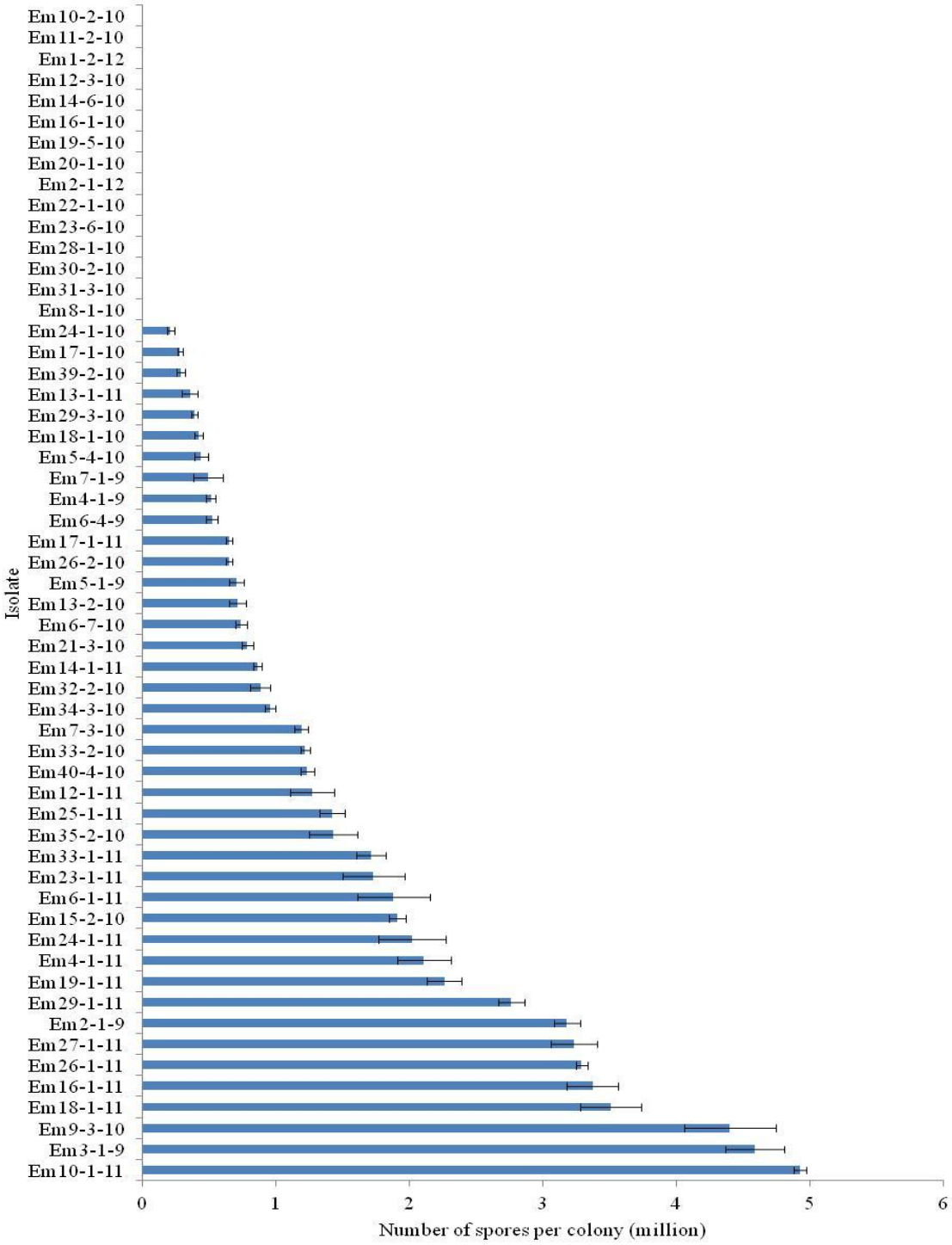

Figure 11: Average of spores per colony of different isolates of *E. mespili*.

The analysis of variance (ANOVA) showed a significant variation of sporulation of the 56 isolates of E. *mespili*. (Number of observation= 224, F value= 138.81, DF= 55, P= 0.0001).

Student-Newman-Keuls Test generated different groups of isolates depending on the number of spores per single culture. Cluster analysis has revealed the presence of two main groups. Group A, with high rate of sporulation (from 1,710,000 to 4,925,000 spores per pure culture), and a group B with a number of spores below 1,430,000 (Figure 11).

The group B was divided into two subgroups. The first division of isolates did not produce pores on PDA media, and the second subgroup of isolates had a low level of sporulation. The rate of conidia production per colony was between 0.2×10^6 and 1.4×10^6 spores per colony.

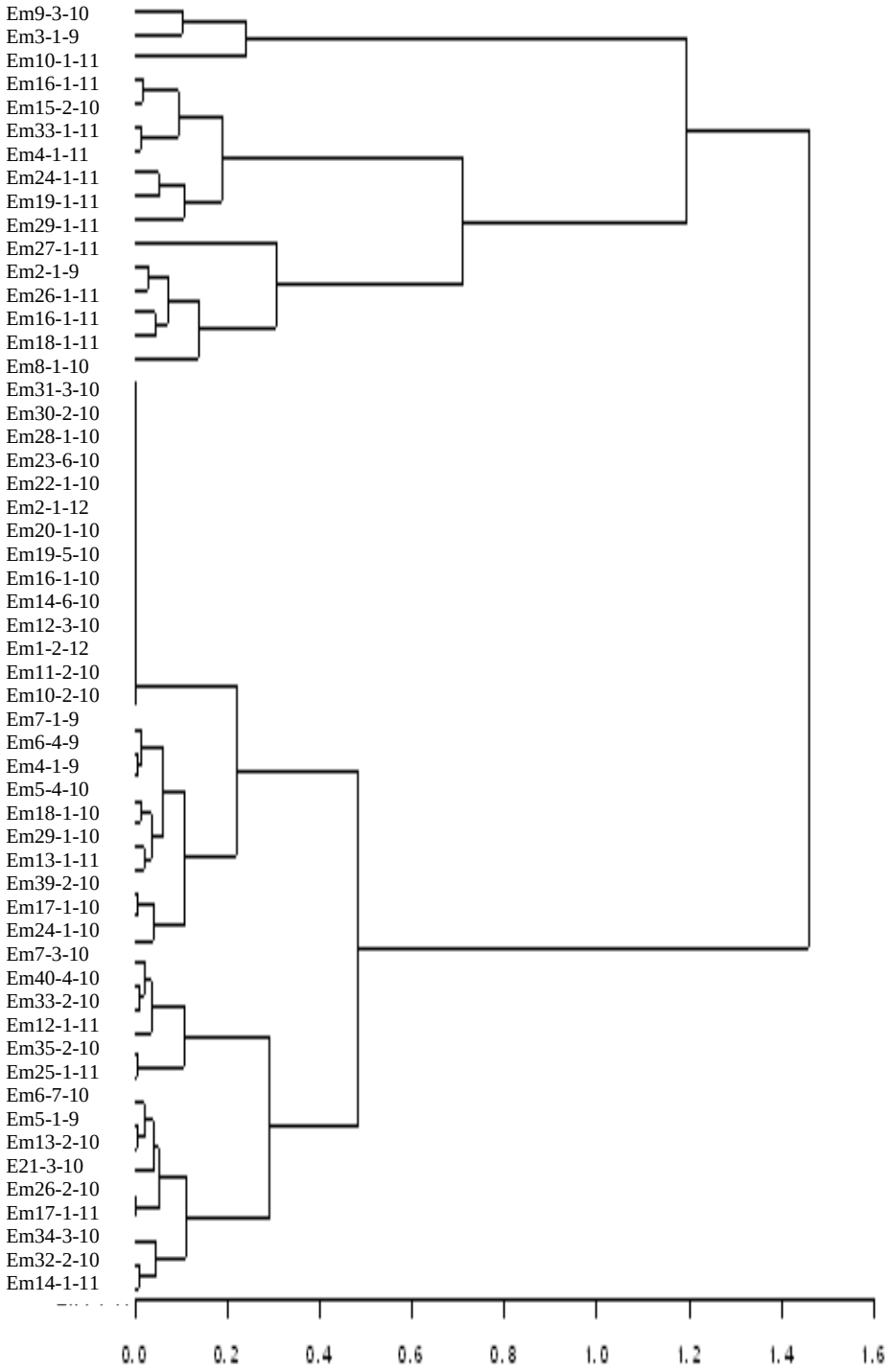

Figure 12: Cluster analysis of sporulation of the tested isolates.

Based on these results, the relationship between the morphological and the degree of sporulation of pure cultures of *E. mespili* was determined.

The coefficient of correlation was 0.21914 between growth rate and sporulation rate. Therefore, the number of spores produced per single colony was not related to its size (Figure 13). Similarly, the color of the colony did not explain the degree of sporulation of isolates.

The geographic distribution of isolates did not determine the degree of the sporulation of *E. mespili*. Different isolates from the same location produced significantly different number of spores.

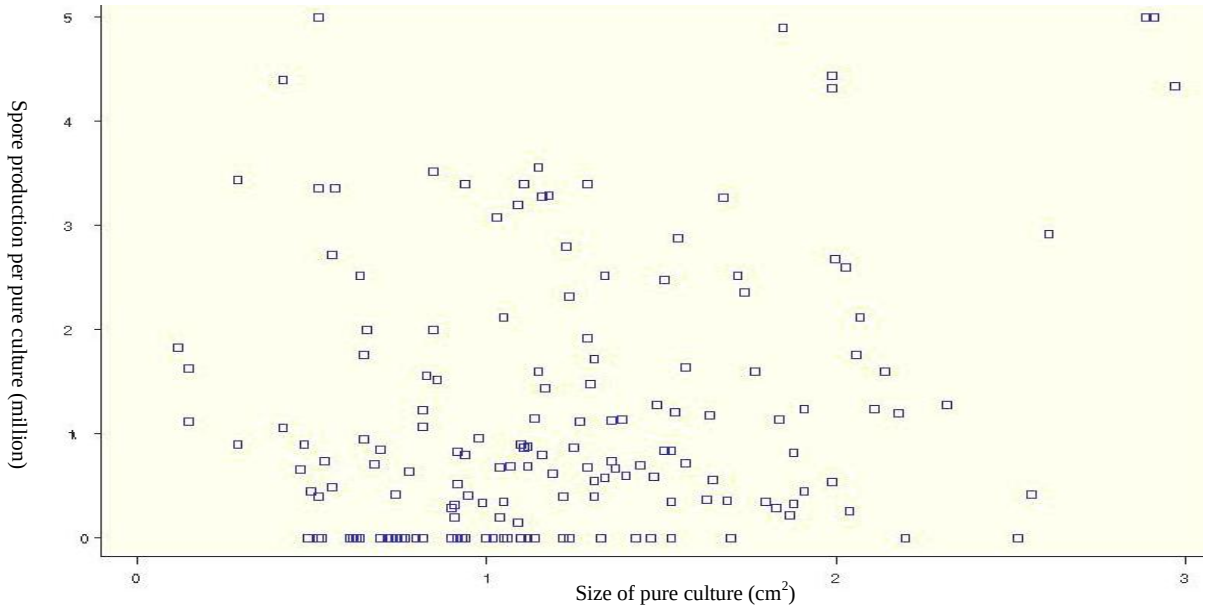

Figure 13: Correlation between the size of pure culture and sporulation.

3.4 DNA extraction

DNA extraction was the critical step in molecular analysis, because studying genetic diversity (RAPD and AFLP) is based on a good quality of extracted DNA. There was no example of DNA extraction of *E. mespili* in the literature. For this reason, different standard protocols were tested in order to optimize DNA extraction of this fungus. This step alone took several months.

DNeasy Plant Mini kit (Qiagen) was the easiest and fastest method to extract DNA. The protocol takes between 1 to 2 hours. In the case of *E. mespili*, the kit provided high quality DNA, but the yield was too low. The average of concentration obtained was 20 ng/µl. This amount is acceptable to run RAPD. However, AFLP required a concentration of DNA between 500 to 1000 ng/µl. DNeasy Plant Mini kit (Qiagen) was not the optimum protocol to be used in the case of *E. mespili*.

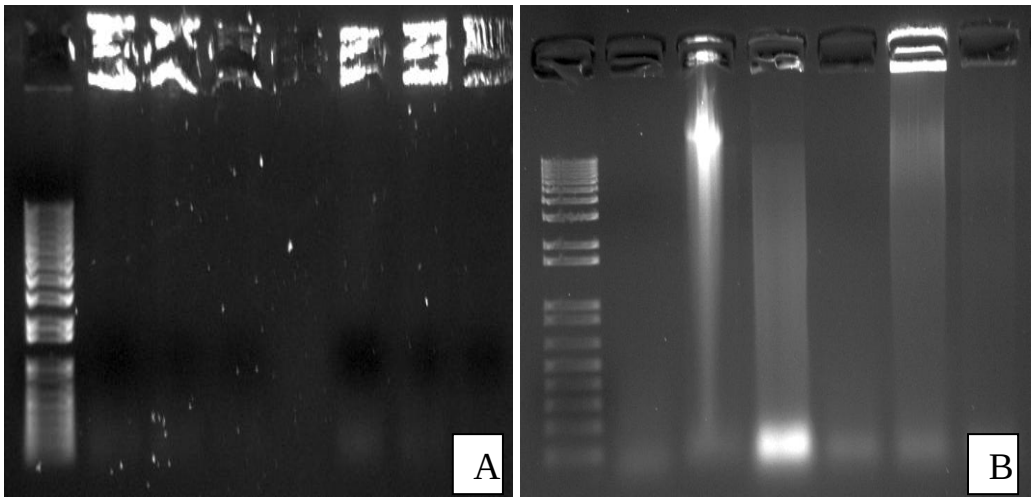

Figure 14: Migration of DNA on 1.2% agarose gel.
A) High molecular weight fixed in the wells.
B) Appearance of smear in 1.2% agarose gel.

The use of CTAB (CTAB1, CTAB2) and SDS protocols did not solve the problem of DNA extraction either. The DNA obtained was not of good enough quality or quantity. For most isolates, DNA concentration was between 20 to 100 ng/µl. The ratio 280/260nm was below 1.7. This low concentration maybe is due to the fact that *E. mespili* produced a high concentration of polysaccharides on PDA. Those compounds co-precipitate with DNA in isopropanol or absolute ethanol. On 1.2% agarose gel, DNA loaded in wells of the gel did not migrate through the gel (Figure 14A). The high molecular weight was fixed in the wells, due to the presence of polysaccharides or proteins. The other problem that we faced in those protocols was the presence of smeared bands that indicate DNA degradation during the extraction (Figure 14B).

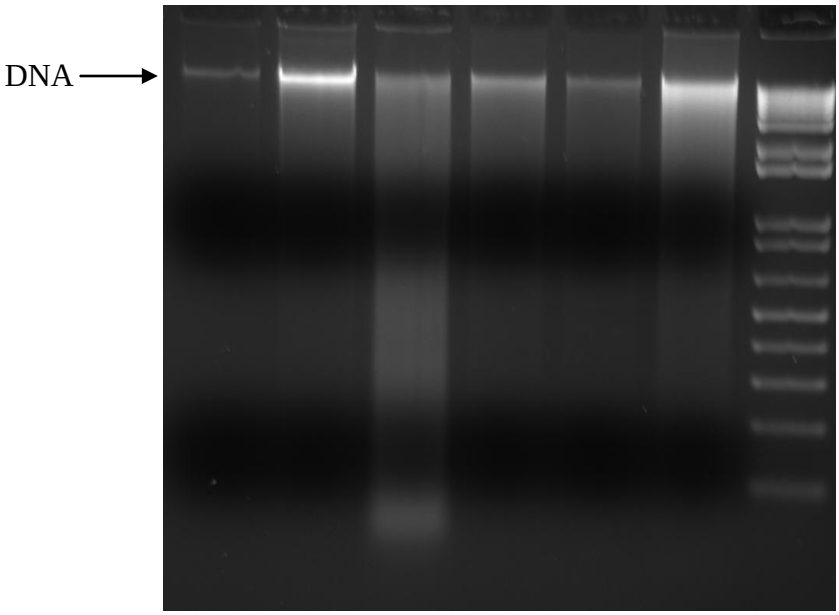

Figure 15: High molecular weight on 1.2% agarose gel.

A good quality and quantity of DNA was isolated by using the modified CTAB/SDS protocol. The use of lyophilizalion instead of liquid nitrogen and the addition of 0.8mg of proteinase K to

the isolation buffer (CTAB/SDS) helped to overcome the problem of DNA degradation. The concentration of the majority of isolates was above 1µg/µl. The ratio 280/260 was between 1.85 and 1.95. A high concentration of 5M potassium acetate added to the isolation buffer followed by incubation on ice precipitated the polysaccharides. The supernatant contained DNA was precipitated with isopropanol. On 1.2% agarose gel, a highly molecular weight band was clearly appeared indicating a good quality of DNA (Figure 15).

3.5 Genetic diversity of *E. mespili.*

3.5.1 Random Amplified Polymorphic DNA (RAPD)

Fragments of genomic DNA were amplified by using RAPD-PCR. All primers tested generated amplification bands from 300bp to 2000bp. The number of bands scored was between 8 to 15 bands per primer.

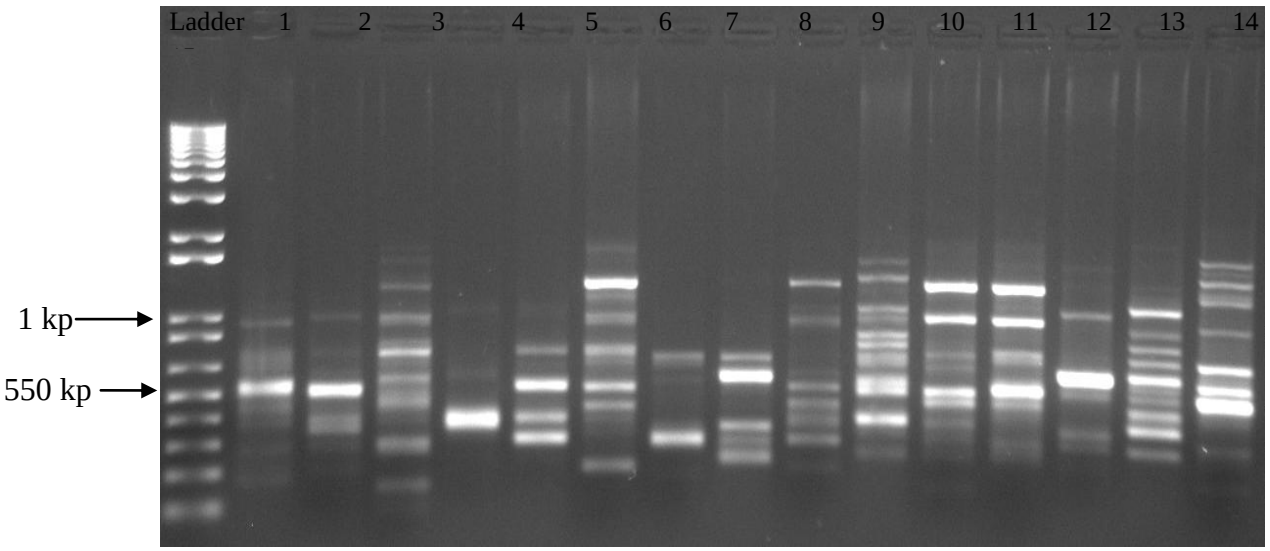

Figure 16: Ethidium Bromide stained 1.2% agarose gel of RAPD markers (primer 21) of fifteen isolates.

RAPD fingerprints contained bands common to all isolates tested. While other amplified bands were absent in some isolates. Those bands determined a high level of polymorphism amongst the 47 isolates tested. Each tested primer has revealed a set of major amplicons that are shared with all isolates of *E. mespili* (Figure 16).

The Primer P21 revealed a reproducible spectrum of amplicons ranging between 400 and 2000bp. The amplicon 550bp was common in most of the isolates tested. Identical sizes of bands were assumed to have identical sequence. The rest of amplicons were polymorphic bands (Figure 16).

Cluster analysis showed an extensive heterology among isolates. It has determined that most isolates were genetically different. The RAPD profile was used to determine the binary matrix of similarity between each pair of isolates, presence (1) or absence (0) of the band. The dendrogram was obtained by cluster analysis of the similarity matrix using the unweighted pair group method with arithmetic means (UPGMA) (NTSYS-pc 2.0) Software.

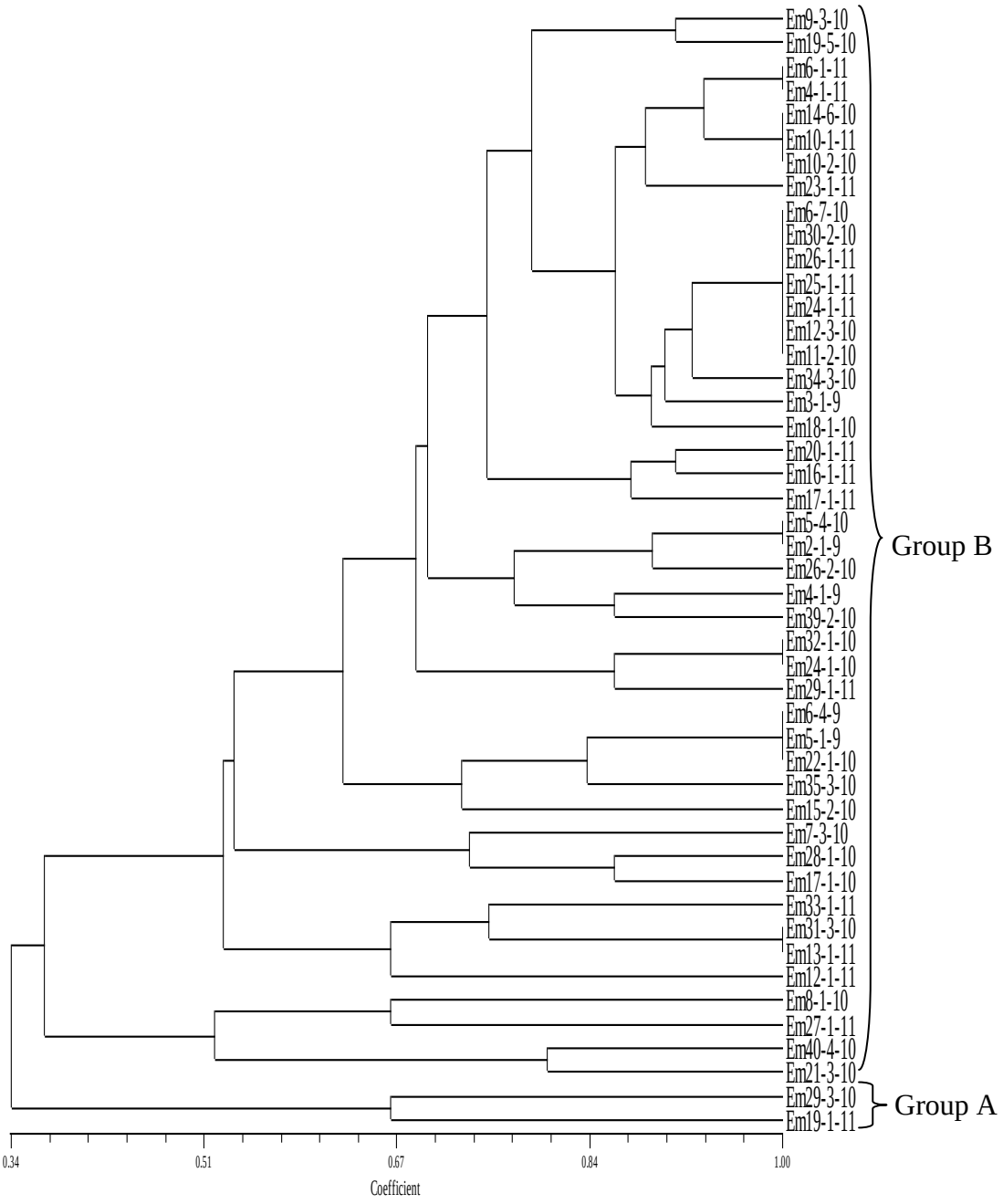

Figure 17: Dendrogram of isolates of *E. mespili* based on RAPD markers.

The genetic similarity was calculated from data of polymorphic bands, based on simple matching coefficients. Values of the similarity coefficients were ranged from 0.34 to 1. The isolates with the value of similarity 1 were 100% identical.

The dendrogram (Figure 17) revealed the presence of two different groups of isolates. Group A, with two isolates Em29-3-10 and Em19-1-11, and group B, with the rest of isolates. The degree of similarity between the two groups was 34%.

The group B contained two subgroups. Group B1 (Em8-1-10, Em27-1-11, Em40-4-10, Em21-3-10), and group B2, with the rest of the isolates. The degree of similarity between the two subgroups was 37%. Within the subgroup B1, the similarity level was above 50%.

3.5.2 Amplified Fragment length Polymorphism (AFLP)

All combinations of primers used in this analysis successfully amplified the digested DNA fragments. The number of amplicons was between 25 and 31 bands. The sizes of those fragments were from 25bp to 12000bp (Figure 18).

The hierarchical clustering revealed a high level of polymorphism, more important than the one shown by RAPD. Only 16% of amplified bands were monomorphic, and 84% were polymorphic. This level of polymorphism was due to the absence of bands in some isolates. The bands that were common in most of isolates were 25bp, 50bp, 125bp, 450bp and 2500bp.

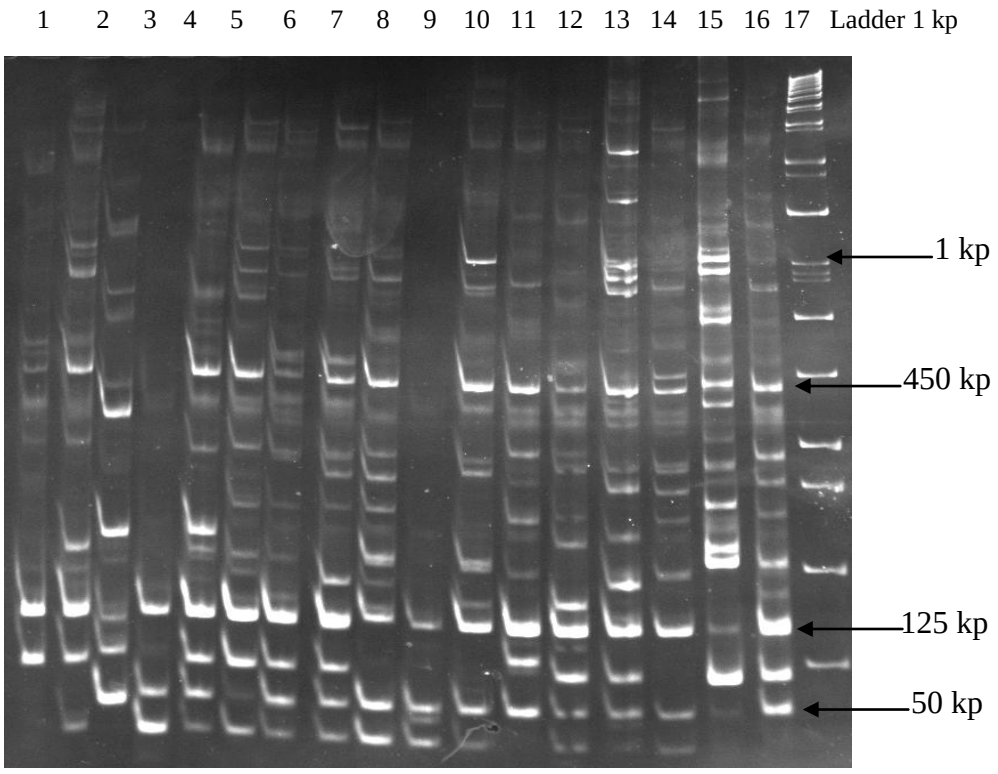

Figure 18: Ethidium Bromide stained 6% acrylamide gel of AFLP markers of seventeen isolates of *E. mespili.*

Genetic similarity among isolates was used to do cluster analysis with UPGMA. The 47 isolates were divided into two groups. Group A with two isolates (Em6-7-10 and Em10-1-11), and the group B with the rest of the isolates. The level of similarity between those groups was 12%. Isolates Em18-1-10 and Em5-4-10 had only 14% of similarity with the rest of the group B. Those isolates had a similarity coefficient ranging between 0.34 and 0.86. Isolates with the coefficient 1 were identical (Em23-1-11 and Em13-1-1, Em5-4-10 and 18-1-10, Em25-1-11 and 17-1-10) (Figure 19).

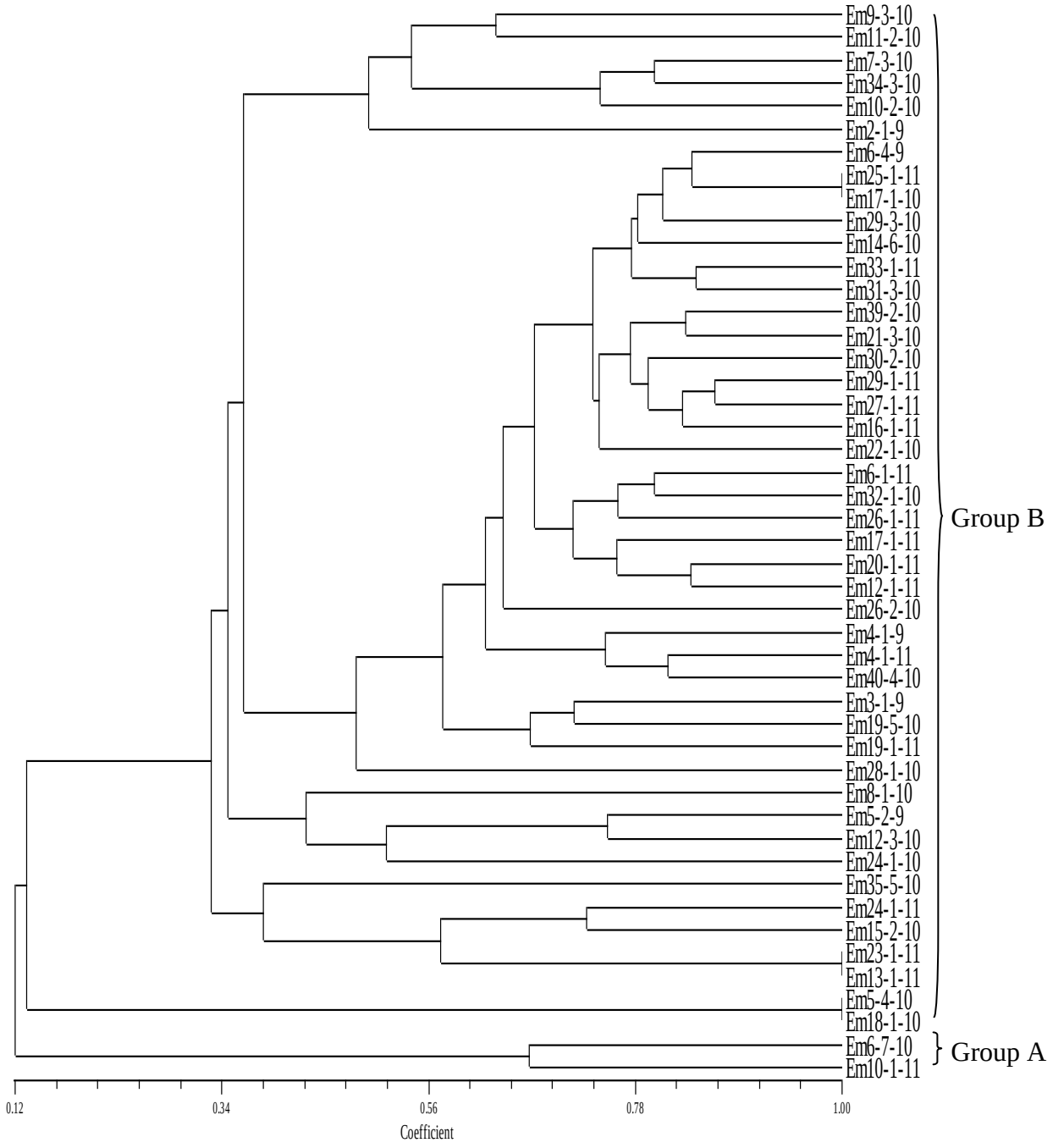

Figure 19: Dendrogram representing the diversity of forty-seven isolates of *E. mespili* based on AFLP markers.

3.6 Pathogenicity tests

3.6.1 Detached leaves

Both inoculation experiments on detached leaves carried out on May 11th and August 5th 2011 did not produce symptoms on detached leaves.

For the test done on September 2012, we tried to overcome the problems above. Conidia were taken from -80°C, grown in PDA media one time, and then harvested after one month. The concentration of spores was increased from 8x10^4 to 1x10^5 conidia per ml. the assessment was done after 12 days.

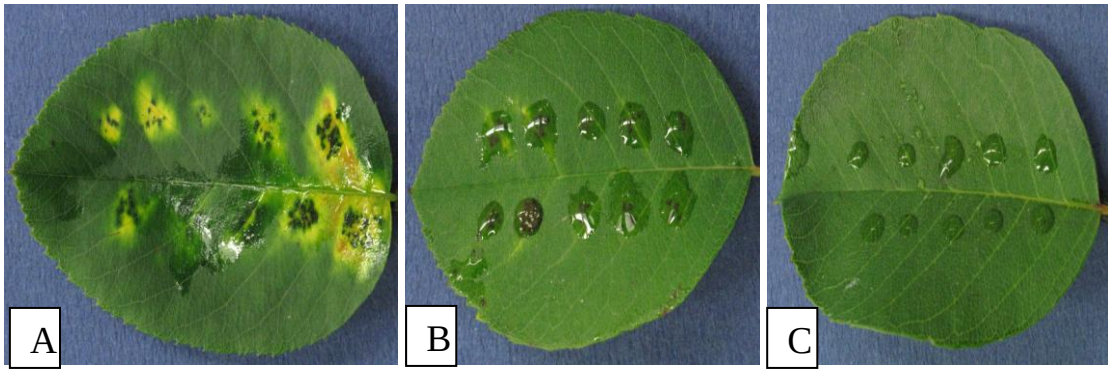

Figure 20: Different levels of *E. mespili* infection on detached leaves fifteen days after inoculation with the fungus

A) High level of severity
B) Moderate level of severity
C) No symptoms

Few of the isolates tested were able to infect Saskatoon leaves (Figure 20). The analysis of Variance has shown significant differences of severity among the tested isolates (F value =4.49, DF=31, P-0.001, Number of observation=96).

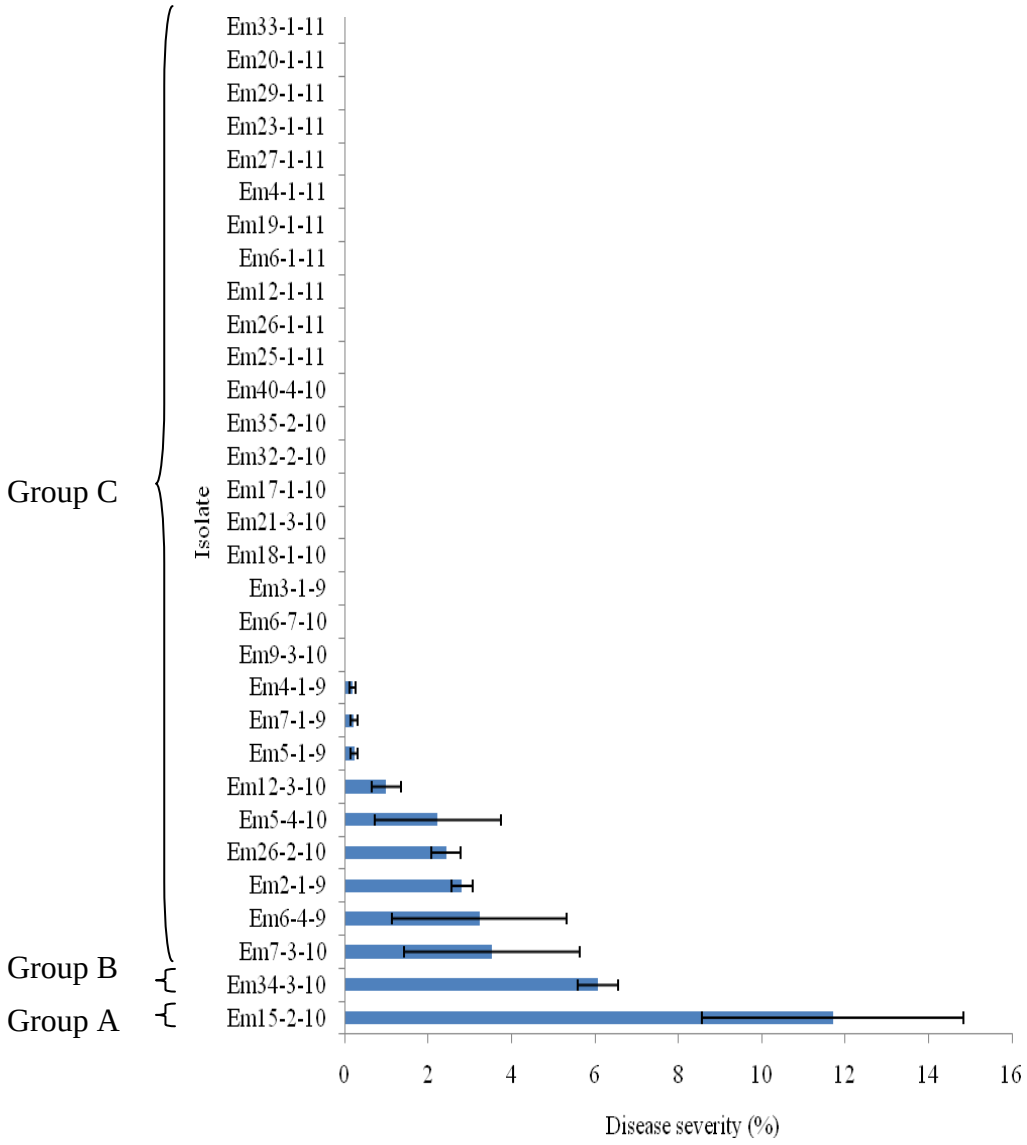

Figure 21: Disease severity of different isolates of *E. mespili* on detached leaves of smoky cultivar.

The analysis of variance has revealed the presence of two main groups. Group A, represented by one Isolate (Em15-2-10) with the highest level of disease severity 12.02%, and group B,

contained the rest of isolates tested, with disease severity below 6.1%. The isolate Em15-2-10 was considered as the most aggressive isolate.

The group B was divided into two subgroups. The first division of isolates did not infect Saskatoon leaves (severity=0), and the second subgroup had isolates with disease severity between 0.19% and 61% (Figure 21).

3.6.2 Saskatoon seedlings

In the experiment June 2011 no infection occurred. On December 2011, in order to mimic natural infection conditions, we used a conidial suspension from the first generation in the inoculation experiment. Only 10 isolates from 2011 were used in this experiment (Em6-1-11 / Em4-1-11 / Em10-1-11 / Em16-1-11 / Em17-1-11 / Em19-1-11 / Em23-1-11 / Em25-1-11 / Em13-1-11 / Em33-1-11). The rest of isolates were not ready at that time for inoculation. The inoculum was collected from colonies after only one transfer on PDA. The conidia were isolated from samples of infected leaves, germinated on PDA, a single spore selected and grown on a new PDA plate. The conidial suspension was collected and used for inoculation directly. The symptoms of infection appeared where an abrasive was used as pre-treatment.

The symptoms appeared as necrotic spots, surrounded by a chlorotic area. However no acervuli were observed (Figure 22).

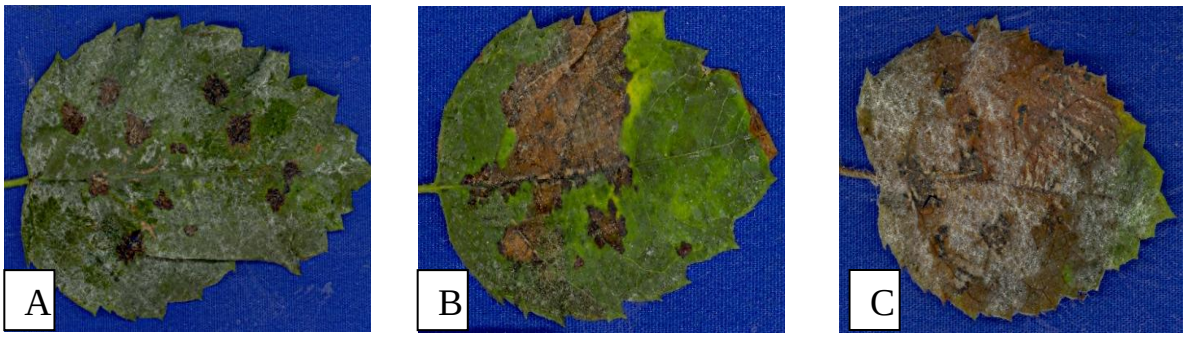

Figure 22: Different levels of symptoms of *E. mespili* on seedlings of Martin cultivar thirty days after inoculation. A: Weakly aggressive isolates, B: Moderately aggressive isolate, C: Highly aggressive isolate.

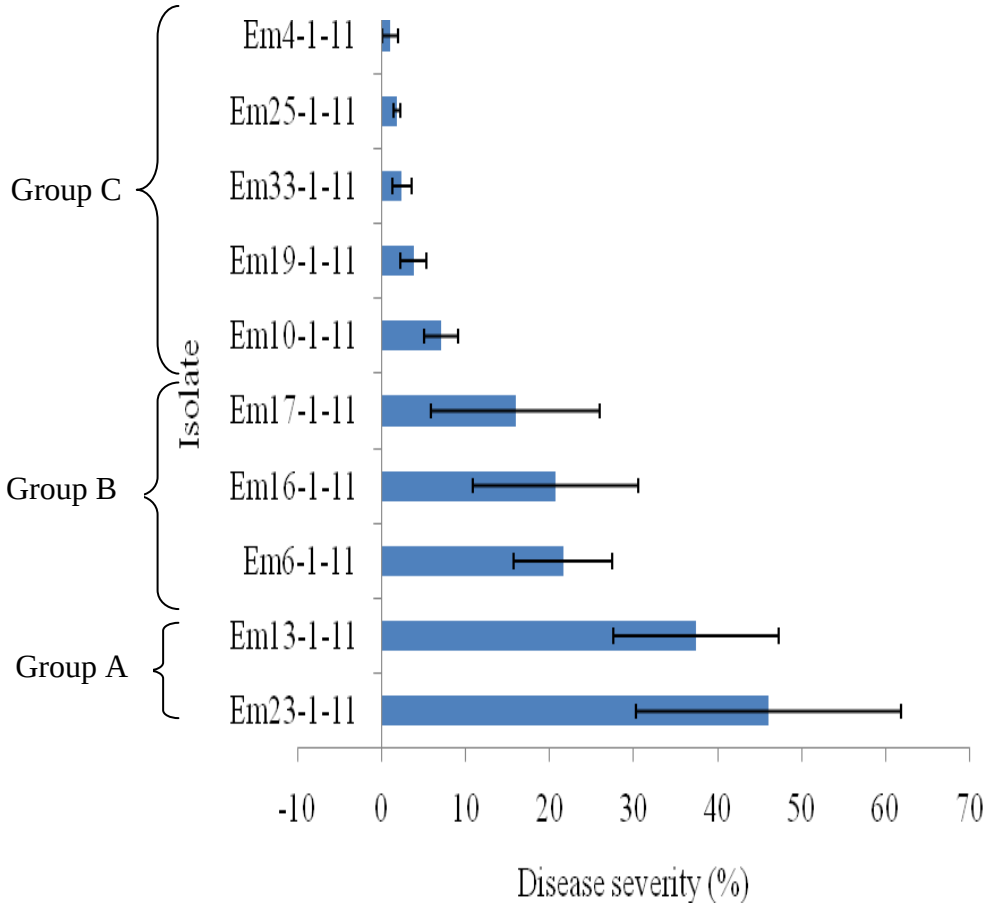

Figure 23: Severity of different isolates of *E. mespili* on saskatoon seedlings.

The severity of infection was different amongst the isolates tested. Statistical analysis using GLM has distinguished between three groups of isolates regarding the aggressiveness (F value=2, DF=12, P=0.819, Number of observation=33).

The first group that is highly aggressive, contains (E23-1-11 / Em13-1-11), with disease severity of 46.09% and 37.37%, respectively. The second group had moderately aggressive isolates (Em6-1-11 / Em16-1-11 / Em17-1-11 / Em29-1-11), with disease severity between 15.96% and 29.70%. . The third group contained weakly aggressive isolates (Em4-1-11 / Em10-1-11/ Em19-1-11 / Em25-1-11 / Em33-1-11), with a low level of disease severity (0.96% to 7.02%) (Figure 23).

4 GENERAL DISCUSSION

Entomosporium mespili is a hemibiothrophic fungus. Its growth on artificial media is limited and very slow. The life span of this pathogen on petri dishes is between 45 to 60 days. Stowell and Backus (1966) determined that the maximum growth of *E. mespili* on PDA is 2.5 mm per week over 2 months.

Among the tested media, PDA remains the best one to grow *E. mespili in vitro* (Piehl and Hildebrand 1936) with faster and more uniform development of the fungus than on other media. Several other media, including those amended with saskatoon berry extracts, did not improve the growth of *E. mespili*, as compared to PDA. Conidia of *E. mespili* germinate and produce white hyphae on the surface of leaves. The colonies grow and become darker with time. The results of *in vitro* growth on PDA were similar to those obtained by Park et *al.* (2011).

The color and size of pure cultures were different among isolates. This variation was not related to the geographic origin of isolates. Some isolates produced white mycelium on the top of the opaque compact mass. The variations in color and size were probably due to differences in metabolism of the isolates on PDA. More in-depth analysis is required to better understand these variations in morphology.

The sporulation on PDA was better than on natural leaves of the saskatoon plant. *E. mespili* produced many white acervuli on PDA with each acervulus containing large numbers of spores. In nature, symptoms of the disease are characterized by the presence of a limited number of black acervuli, and a limited number of conidia. The morphology and physiology of *E. mespili in vitro* apparently are different than in nature. On PDA, it is probably the glucose that stimulates

the sporulation of the fungus (van der Zwet and Stroo 1985). However; it is not clear why the increase in sporulation is not matched with an increase in disease after inoculation.

The degree of sporulation also showed a high variation among *E. mespili* isolates. Three categories of isolates were determined, isolates without sporulation, those with a medium level of sporulation, and those with a high level of spore production. However, the level of sporulation was not correlated with the color, the colony size, or its geographical origin.

Van der zwet and stroo (1984) suggested the use of casein-sucrose-thiamine-based medium to increase the sporulation of *E. mespili*. According to these authors, this medium increases the number of spores produced by the fungus up to 200-fold more than on PDA. In this study, *E. mespili* produced more mycelium than spores on casein-sucrose-thiamine. In most cases, the fungus did not produce spores at all on this medium. Perhaps the addition of fructose in the casein-sucrose-thiamine-based medium promoted the mycelia growth over the sporulation.

Liquid media such as Potato Broth are useful to maximize the production of mycelium, while providing more nutrients for the development of the pathogen. Obtaining enough mycelium was essential for DNA extraction. These results are opposite to those revealed by Piehl and Hildebrand (1936), who suggested that the growth of *E. mespili* was not good in the liquid media they tested. The explanation of these differences in results may be due to the fact that Piehl and Hildebrand (1936) used glass tubes of 15ml. In this study, 250 ml flasks were used for liquid media, with the intent to provide the fungus with enough aeration and nutrients for a faster and better growth.

Successful extraction of good-quality DNA was a critical point in this study, because molecular studies are based on obtaining good quantity and quality of DNA. Based on the literature search, no molecular studies were performed on *E. mespili*. Therefore, this research used several protocols to optimize the DNA extraction. After comparison of several protocols and variations, the best protocol to extract good DNA in the case of *E. mespili* is the use of a mixture of CTAB and SDS, with the use of lyophilized tissue to improve the extraction process. The addition of proteinase K (enzyme that degrades proteins) is important to avoid the degradation of DNA during incubation. Also, the fungus produces a high level of polysaccharides. To overcome this problem, the polysaccharides were precipitated first in a high concentration of salt, followed by a separation with phenol:chloroform:isoamyl alcohol(25:24:1).

Molecular markers RAPD and AFLP produced evidence of a high level of polymorphism among the isolates of *E. mespili*. This polymorphism is not related to the geographical origin of the isolates. However, such genetic differences are in line with the abundant morphological and sporulation variations in this pathogen's populations. AFLP is more accurate and more reproducible than RAPD when running genetic studies. It revealed several polymorphic bands among the tested isolates. The amplified bands can be sequenced and might pinpoint potential genes that are responsible for these variations.

For the pathogenicity tests, many hypotheses could be presented to explain the absence of symptoms in both inoculation experiments on May11th and August 5th 2011:

-The detached leaves used in those experiments were kept in the fridge at 4°C before being inoculated. So, it is possible that the leaves were too old to be inoculated.

-The initial stock of conidia was collected after three transfers on PDA, and then conserved at -80°C for six to nine months. Those conidia were then grown in new PDA to prepare new inoculum. Van der zwet and Stroo (1984) have reported that *E. maculatum* lost pathogenicity rapidly and became avirulent after three transfers on artificial media.

-Growing the fungus *in vitro* proved that natural infection is totally different from artificial infection. The loss of virulence is a fact in the case of *E. maculatum* after transfers *in vitro*.

In the inoculation experiment in September 2012, the severity of infection was different among isolates. On detached leaves, the isolate Em15-2-10 was the most aggressive one. The level of disease severity it induced was significantly different from the rest of the isolates.

No infection occurred in the pathogenicity tests on saskatoon seedlings on June 2011. The reasons of this failure could be related to the age of inoculum. The conidial suspensions used were obtained after more than one transfer in PDA plates. On detached leaves, only few isolates were able to reproduce symptoms of the disease. On seedlings, the isolates Em13-1-11 and Em23-1-11 were highly aggressive after pre-treatment with an abrasive.

The infection on both detached leaves and seedlings did not complete the fungus' cycle after four weeks. The pathogen did not produce acervuli on the surface of the infected leaves. Only chlorosis and necrosic were visible. The highly aggressive isolates Em13-1-11, Em23-1-11, and Em15-2-10 can be very useful in further studies on this pathogen, such as comparing expression of potential pathogenicity genes in these in comparison with avirulent isolates.

CONCLUSION

Studying *E. mespili* is challenging due to its hemibiotrophic mode of life and to the fact that few studies were done on it before. Growing the fungus *in vitro* takes time. Another major problem in studying *E. mespili* is the change in its physiology when grown on artificial media.

As a result, it is difficult to study the pathogenicity of this pathogen in accurate terms. Simply applying infected leaf material on fresh leaves provides more symptoms, but the amount of inoculum propagules is not known and this method may let other pathogens interfere with the results.

In order to study the genetic diversity of this pathogen, an important amount of good-quality DNA is usually required for several techniques. The production of mycelium in liquid media is essential to get good quality and quantity of DNA. However, the media usually stimulate the production of high concentrations of polysaccharides. On PDA plates, the growth of the opaque mass of colonies is probably due to the production of polysaccharides. In this study, we succeeded in optimizing DNA extraction from *E. mespili* by modifying an SDS extraction protocol. The use of proteinase K followed by a first precipitation of polysaccharides in high concentration of salt increases the quantity and the quality of DNA.

A high level of polymorphism among forty-seven isolates was revealed after using two molecular markers. Genetic distance between isolates might be more related to the medium used. More in-depth genetic analyses are recommended to better understand the high level of diversity in this pathogen.

On PDA, the degree of sporulation was significantly higher in comparison with natural production. However, this did not translate in a higher level of symptoms after artificial inoculation of saskatoons. *E. mespili* seems to lose pathogenicity when it is grown on PDA and probably on any artificial medium. Nevertheless, we managed to run some tests. The isolates that we tested had different levels of pathogenicity. Isolates Em13-1-11, Em23-1-11, and Em15-2-10 were highly aggressive on saskatoon leaves compared with the rest of the tested isolates, while other isolates did not produce symptoms.

To further optimize the inoculation process of saskatoon leaves with *E. mespili*, we recommend multiplying spores naturally on healthy saskatoon seedlings before using them for inoculation. This would prevent the use of artificial media that seem to reduce this fungus' pathogenicity.

For saskatoon growers, the fact that the population of *E. mespili* is highly polymorphic, it is recommended to alter the fungicides used to control the ELBS, and reduce the number of chemical treatments, in order to prevent the risk of resistance to those chemical molecules.

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