DIPLOMA THESIS

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BUDAPEST

Fitness and pathogenicity of *Venturia inaequalis* isolates

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TABLE OF CONTENTS

FIGURES

TABLES

USED ABBREVIATIONS

Abbreviation	Meaning
AI	Artificial Inoculation
Avr	Avirulence
avr	Virulence
C	Control
Df	Degrees of freedom
DLA	Detached Leaf Assay
DNA	Deoxyribonucleic acid
EU	European Union
GfG	Gen-for-gene relationships
GMO	Genetically modified organism
LG	Linkage group
MAS	Marker-assisted selection
PAPMs	Pathogen-associated molecular patterns
PDA	Potato Dextrose Agar
PTI	PAMP-induced immunity
qPCR	Real-time polymerase chain reaction
QoIs	Quinone outer binding site of the cytochrome bc1 complex Inhibitor
QTL	Quantitative trait loci
R	Resistance
ROS	Reactive oxygen species
SSR	Simple sequence repeat
T1	Treatment 1
T2	Treatment 2
T3	Treatment 3
UNECE	United Nations Economic Commission for Europe

1. INTRODUCTION

Venturia inaequalis (*Cke*.) Wint. is the causal agent of apple scab disease, which is the main fungal disease of apple (*Malus* x domestica), especially in production regions where moist conditions and cool temperatures prevail, such as northeastern America, Europe, South America, and Asia (Carbone, 2017). This pathogen has shown broad geographic distribution and well-established populations that can be found continuously in almost every country where apple is cultivated, with the exception of West Australia (González-Domínguez et al., 2017; McKirdy et al., 2001).

One of the major challenges of the twenty-first century is to provide high quality crops for its increasing population. In order to meet this demand, farmers must produce more crops in line with the market requirements which include acceptable fruits appearance. This has led to an intensive pesticide application every growing season during the last decades in order to control the spread of the apple scab pathogen (Zelmene et al., 2022)

Despite the importance of chemical control in the food system, its persistent overuse has caused a number of adverse effects. Thus, several strategies should be applied to provide high-quality fresh products to future generations taking into consideration economic viability and ecological sustainability (Kaur and Kaur, 2020). These strategies are based on the Integrated Disease Management, including resistant cultivars, cultural practices, and chemical control (Ward, 2012).

In the last decades, several attempts have been made for growing resistant varieties and in the last 20 years more than 200 scab-resistant cultivars have been released. Even though these efforts, apple industry still relies on monogenetic resistance, and the *Rvi6* resistance gene is the basis of almost all contemporary apple scab-resistant cultivars (Ignatov and Bodishevskaya, 2011).

Relying on a single *R*-gene represents a risk on the long run due to the evolutionary pressure on a given *V. inaequalis* population that results in genetic mutations of the pathogen as adaptation mechanism, which might lead to emergence of new races able to avoid recognition by the plant (Masny, 2017). This is common in monoculture orchards where there have been documented cases *of V. inaequalis* overcoming apple scab resistance Bus et al., 2011; Ignatov and Bodishevskaya, 2011).

For that reason, the scientific community encourage the use of combination of different *R*-genes for new developing new cultivars, and/or replanting traditional cultivars as a long- term strategy to produce more eco-friendly, robust and resilient apple fruits (Höfer et al., 2021). However, more data is needed to better understand the evolutionary processes of the apple scab pathogen guided by its ability to persist successfully in its host (fitness-cost) (MacHardy et al., 2001).

2. OBJECTIVES

The main goal of this thesis work is to quantify different fitness parameters of four *V. inaequalis* races and to better understand the relationship between these parameters. The race isolates are originated from four scab race indicator cultivars, 'Gala' (no *R-gene),* 'Golden Delicious' *(Rvi1),* 'Geneva' *(Rvi3,* complex), and OR45t132 *(Rvi5),* thus representing the corresponding pathogen races. In the present work, we will evaluate parameters that play a major role in the pathogen's survival ability through time (fitness), including vegetative growth and reproductive success, and also the ability to infect different apple cultivars *in vitro* (pathogenicity).

The following steps will be carried out, including measuring the fungal cultures' growth under different lab conditions and the pathogen's ability to produce asexual conidia spores *in vitro* in order to evaluate the vegetative growth and reproduction success of the race isolates, respectively. Furthermore, *V. inaequalis* pathogenicity will be assessed by observing microscope (structures from the interaction between fungus– apple) and macroscope (symptoms range) responses on apple leaves infected *in vitro.* As an outcome of this work, we will have information about the ability of *V. inaequalis* isolates to grow, reproduce, and infect different susceptible and resistant apple cultivars *in vitro.*

3. LITERATURE REVIEW

Apple (*Malus* x domestica) belongs to the genus *Malus*, a member of the Maloideae subfamily of the family Rosaeae. Its center of origin is Asia (current Republic of Kazakhstan) (Musacchi and Serra, 2018). Apple has been cultivated for at least 3,000 years and genetic evidence suggests that its primary ancestor is *M. sieversii* (Davies et al., 2022).

Apple is the third most important fruit crop worldwide after banana and watermelon. It has great value for its taste, nutritional value, and availability throughout the year. FAO (2021) reports its production reached 88.3 million tons in 2019 (FAO, 2021). In 2020, apple was one of the most traded products ($7.39B), being China, Italy, United States, New Zealand, and Chile main exporters; while Germany, Vietnam, United Kingdom, Russia, and Thailand were main importers (OEC, 2020).

Commercial production of apple is spread in a wide range of growing conditions, including temperate, subtropical, and tropical climates (latitudes between 25° and 52°). Over 63 countries produce a large number of apple cultivars, and the European Apple Inventory alone includes around ten thousand cultivars. The majority of world apple production is based on mutations from original cultivars such as 'Gala', 'Golden Delicious' or 'Fuji'. In case of apple, agricultural breeding and management techniques have aimed to increase yield and quality for many years by selecting traits of interest present in specific cultivars (Musacchi and Serra, 2018).

Since the beginning of the 20th century, apple breeding for disease resistance became a dominant approach. Initially, the breeding focused on apple scab resistance and, to a lesser extent, resistance to powdery mildew. Later, other fungal and bacterial diseases also gained importance, such as fire blight and the juniper rusts, including cedar-apple rust, cedar-quince rust, and cedar-hawthorn rust (Beckerman, 2006).

3.1. Apple scab

V. inaequalis is the causal agent of apple scab disease, which is one of the main fungal diseases of apple, especially in production regions where moist conditions and cool temperatures prevail during the spring months (Carbone, 2017).

3.1.1. Geographical distribution

V. inaequalis has shown broad geographic distribution and well-established populations **(Figure 1)** (González-Domínguez et al., 2017). Apple scab exists in every country where apple (*Malus* x domestica) is cultivated with the exception of West Australia, where the disease has not been established and occasional outbreaks have been eradicated (McKirdy et al., 2001).

The origin and spread of *V. inaequalis* with the domestication of apple has been demonstrated by using simple sequence repeat (SSR). Gladieux (2010) carried out a study by analyzing microsatellite data and suggested that the domestication of apple induced a significant change in the genetic differentiation of populations of *V. inaequalis* in its center of origin. Then, in the course of human migration into new lands, Europeans dispersed domesticated apple which carried the pathogen (Gladieux et al., 2010).

This is supported by the observation that there is more genetic diversity among populations of *V. inaequalis* on *M. sierversii* (the main progenitor of the domesticated apple) in Central Asia than in European populations from *Malus* x domestica and *M. sylvestris* (a secondary crabapple progenitor of the domesticated apple), as well as in European populations than in populations from areas where apples have been more recently introduced e.g. some states in the USA, Japan, and Australia (Bowen et al., 2011).

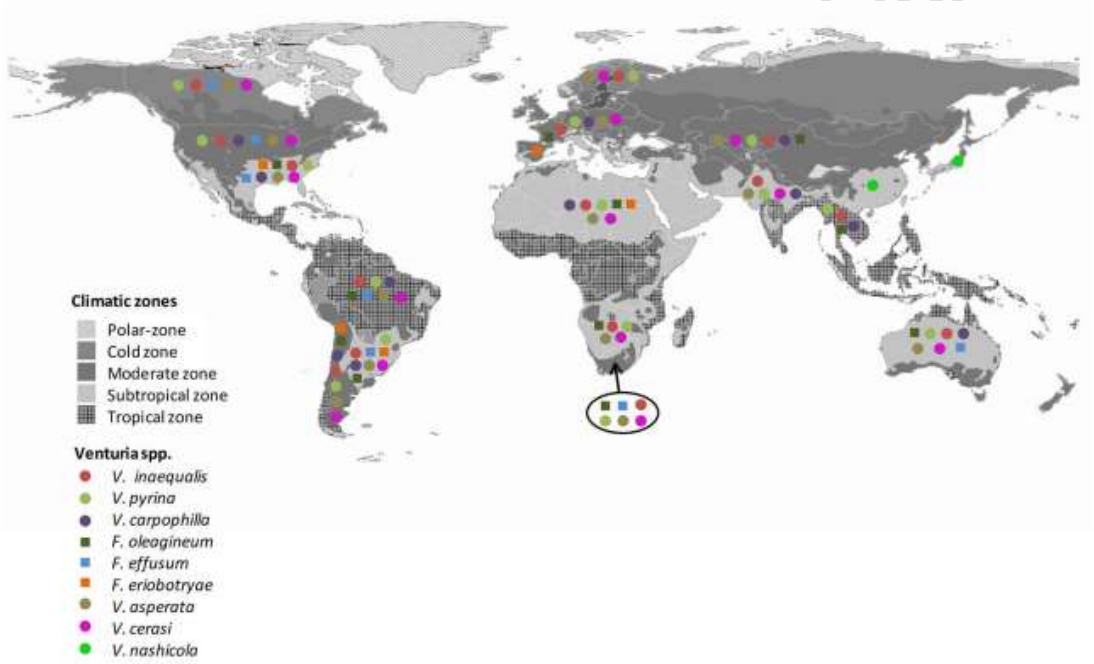

Figure 1. Worldwide distribution of *Venturia spp.* Colors and symbols indicate the presence of the different Venturia spp. in each region (González-Domínguez et al., 2017).

3.1.2. Taxonomy and host range of *Venturia spp.*

Venturia species belongs to the kingdom Fungi, phylum Ascomycota, subphylum Pezizomycotina, class Dothideomycetes, order Pleosporales, and family Venturiaceae (Singh, 2019). These species are typically host specific, infecting one or only a few host species. Many *Venturia* species attack fruit crops including: *V. pyrina* and *V. nashicola* affecting European pear *(Pyrus communis)* and Asian pear *(P. pyrifolia* and P. *ussuriensis),* respectively; *V. carpophila* affecting peach *(Prunus domestica)* and almond *(Prunus dulcis); V. asperata* affecting apple (*Malus* spp.); *V. cerasi* affecting cherry *(Prunus cerasus); Fusicladium oleagineum* affecting olive *(Olea*

europea); F. effusum affecting pecan *(Carya illinoinensis);* and *F. eriobotryae* affecting loquat *(Eriobotrya japonica)* (González-Domínguez et al., 2017).

Several molecular studies have evaluated the phylogenetic relationships among *Venturia* species affecting fruit trees. *Venturia spp.* share a common recent ancestor and have close co-evolutionary development with their hosts (González-Domínguez et al., 2017). It infects only members of the genus *Malus,* excluding the rare formae speciales strains infecting e.g., *Pyrachanta* species (le Cam et al., 2002).

3.1.3. Symptoms

Apple scab was register for the first time in 1819 in Sweden. Its causal agent attacks leaves, petioles, pedicels, flowers, and fruits, but the most visible symptoms occur on leaves and fruits. Twig and blossom lesions have the shape of small scab spots, but they are uncommon and of little importance (Singh, 2019).

Symptoms on leaves include olive green spots which progressively become brown, thicker (3-10 mm), indeterminate margins, and velvety appearance. Lesions are distributed on the upper and lower surfaces of infected leaves, appearing more obvious on the later one. Usually the leaves keep its shape, even though when the lesion develops along the veins or margins the leaf may be disfigured. Heavily infected leaves drop prematurely (Singh, 2019; Ward, 2012).

Symptoms on fruit are similar to those on leaves. When infection occurs on young fruit, the lesions may cause the fruit to become severely deformed. On the other hand, when infection establishes late in season, lesions may not become visible until the apples are in storage. Heavily infected fruit whose pedicel is affected may fall off prematurely (Singh, 2019; Ward, 2012).

3.1.4. The life cycle of *Venturia inaequalis*

Apple scab disease develops in two cycles characterized for producing sexual and asexual spores, both able to infect apple **(Figure 2).** The primary cycle includes the establishing of the disease and the saprophytic stage of the fungus (perfect stage) which occurs on the infected fallen leaves in winter and spring. On the other hand, secondary cycle involves the parasitic stage (imperfect stage) which occurs on the fruits and leaves throughout the growing season (Doolotkeldieva and Bobusheva, 2017; Singh, 2019).

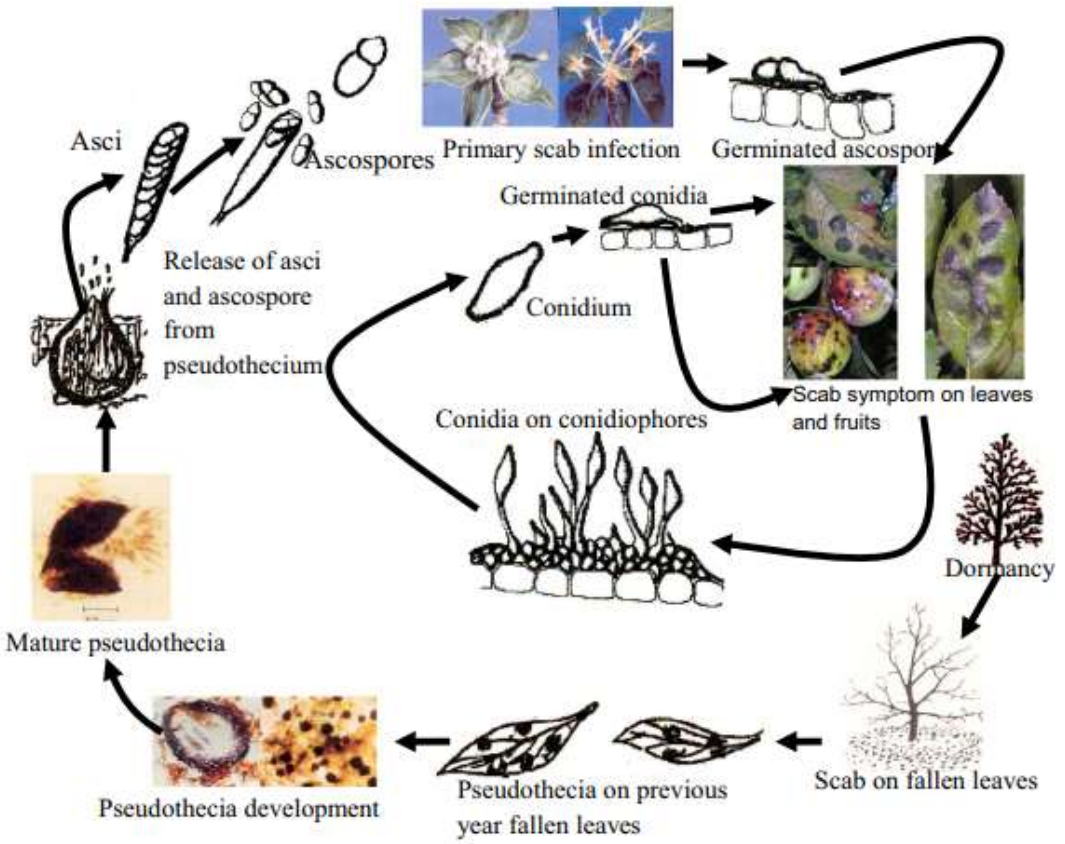

Figure 2. Life cycle of *Venturia inaequalis* (Singh, 2019).

3.1.4.1. Primary cycle

The pathogenic phase of disease starts with germination of sexual spores called ascospores that serve as primary source of inoculum. When leaf fall begins in autumn, the mycelium penetrates the leaf tissues and switches from vegetative to reproductive phase. Then, mycelia of two different mating types produce pseudothecium which exhibits negative geotropism. This directional growth will ensure that ascospores are released towards the atmosphere, and it might be an evolutionary mechanism for inoculum conservation (Gadoury and MacHardy, 1985). *V. inaequalis* overwinters in infected fallen leaves on the ground (saprophytic lifestyle). During autumn, fruiting bodies are formed within pseudothecia and it will continue by late winter and even in early spring (Singh, 2019).

Pseudothecia of *V. inaequalis* are observed as small, black, dome-shaped tubercles. They are located embedded in the leaf tissue and its opening (ostiole) comes through the cuticle. The asci are bitunicate, cylindrical, double walled, and loculus **(Figure 3).** Inside them, ascospores consists of two unequal sized cells for which the term *"inaequalis"* was assigned. An important feature of the ascospores consist of having two walls: a thin outer and a tick elastic inner one, which helps the pathogen to survive the winter (MacHardy, 1996).

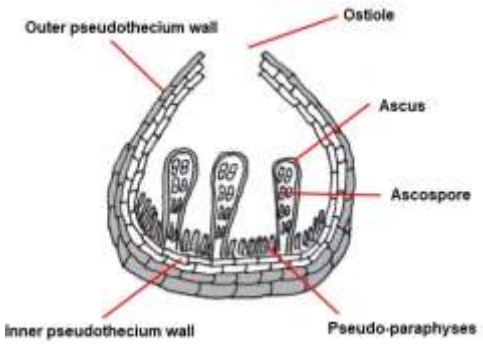

Figure 3. Pseudothecium structure of *V. inaequalis* (Vaishali Patil, 2021).

The ascospores mature when apple buds break and the plant tissue are susceptible. Then, they need moisture from precipitations to be released and also presence of light for optimal discharge (Machardy et al., 2001). Then, they are carried up to 300 meters by wind currents, reaching even neighboring plantations (Carbone, 2017). According to Singh (2019), around 2000 pseudothecium with a total load of about 2 million ascospores may be present on a single leaf. Mature ascospores are discharged over a period of 5–9 weeks when temperature conditions are optimal (10 – 18°C) (Singh, 2019).

In order to establish the infection, *V. inaequalis* needs to attach and penetrate into the host. Therefore, upon contact with a cuticle, the germ tube differentiates into an appressorium and produces sticky, mucilaginous substances composed of proteins and carbohydrates such as β-galactose and N-acetylglucosaminyl residues. They assist the attachment to the surface of the host (Doolotkeldieva and Bobusheva, 2017). Then, the ascospores initiate primary infections and germinate on young leaves, breaking the surface epithelium due to the pressure of the growing mycelium. Ascospores discharge can continue to cause primary infections from bud break until 2 to 4 weeks after petal fall (Ward, 2012).

3.1.4.2. Secondary cycle

When the infection is established, hyphae differentiate into primary hyphae, which then form subcuticular stroma that produce conidiophores (Chevalier et al., 2004). The conidiophores come from subcuticular or intra epidermal mycelium and appear as simple cylindrical shapes or sometimes swollen at the base. Later in the spring, asexual spores called conidia are produced at the tip of conidiophores **(Figure 2)** (Singh, 2019).

Conidia have a smooth appearance, obpyriform or obclavate shape, pale or mid-olivaceous brown in color (Jha et al., 2009). They proliferate continuously and are released by breaking the leaf cuticle. Then, they are washed down in presence of rains, thus, they are able to infect other leaves or fruits (Singh, 2019). At this time, the external symptoms of the disease are visible and the conidial sporulation results in the velvety appearance of the lesions (Doolotkeldieva and Bobusheva, 2017).

Several secondary infections of the conidial stage occur throughout the growing season. Thus, a few initial primary infections can produce hundreds or thousands of secondary infections as the year progresses (Ward, 2012). Conidia are the main source involved in the development of apple scab in summer and early autumn, and each leaf lesion may contain up to 100,000 conidia that may germinate after 2-4 hours under favorable conditions (Singh, 2019).

After the second and third infections, the scab disease reaches its maximum intensity and its further development depends on humidity. During summer, the drought suppresses the development of scab disease due to the conidiophores quickly lose their ability to germinate and infect the leaves. The full development of the parasitic phase of the scab disease ends with the cessation of growth of apple trees, that is when the leaves fall and the fungus returns to a saprophytic mode of life (Doolotkeldieva and Bobusheva, 2017).

3.2. Plant-pathogen interactions

Plant-pathogen interactions are subtle interactions between potential hosts and pathogens to exchange molecular signals that determine a response. Jones and Dangl (2006) proposed a coevolutionary model of plant–pathogen interactions called the zigzag model. The model is focused on how plants attempt to recognize intruders by using pattern recognition receptors that identify molecules associated with pathogens. This is considered as plants' first line of defense against pathogens (Jones and Dangl, 2006).

Pathogen-associated molecular patterns (PAMPs) are shared by a wide range of unspecialized microbes, and they can initiate low-level protective responses called PAMP-induced immunity (PTI). In response of that, pathogens evolve effectors that enhance their ability to cause diseases, often by blocking or suppressing PTIs. Plants then evolve resistance *(R)* proteins that recognize subsets of these effectors either directly or through their action on plant host targets. Therefore, the host range of a particular pathogen is determined by each effector and *R* protein (Deng et al., 2017).

3.2.1. *Venturia inaequalis – Malus* pathosystem

The isolates of *V. inaequalis* exhibit differential pathogenicity on apple cultivars (differential hosts). Historically, the pathogen was categorized into eight physiological races based on those different pathological profiles on apple cultivars. However, Bus et al. (2009) proposed a new nomenclature system by reconsidering the differential host used previously. In their proposal, the old differential host containing more than one *R* genes were replaced with new accession/selection containing only one *R* gene **(Table 1).** The aim of this work was updating the nomenclature system in line with the increasing combinations of genes involved in both race-specific and race-nonspecific recognition by the host (Bus et al., 2009). This work was well accepted in the scientific community due to its standardization, robustness, and easiness to add differential host when a new *R*-gene is discovered in the future (Jha et al., 2009).

The relationship between a host and pathogen is dynamic and its understanding is important to breed resistant cultivars. A complex system *V. inaequalis–Malus* has evolved through time, including the specific interactions of avirulence *(Avr)* genes in the pathogen with resistance *(R)* genes in the host (**Figure 4,** Bus et al., 2011).

The theory of the complementary of genes or so-called gen-for-gen (GfG), proposed by Flor, had its starting point in the 1940s. According to his studies, each resistance gene *(R)* of the host plant has a counterpart in the genome of the pathogen, which determines its pathogenicity (Flor, 1971). In case of apple, the presence of the *Rvi* gene (responsible for scab resistance) allows the recognition of *V. inaequalis* that has the specific avirulence gene *(AvrRvi),* which results in unsuccessful infection by the fungus in this specific apple genotype (Masny, 2017).

GfG relationships determine resistance in the host because of the corresponding gene that conditions pathogenicity in the parasite. GfG relationships in *V. inaequalis-Malus* are based on the segregation of *Avr* genes in the fungus. Since ascospores of the fungus produce haploid mycelium, either the genotype avirulence (*Avr*) or virulence (*avr*) is expressed at each locus (Bus et al., 2011). Therefore, pathogens containing *Avr* genes are avirulent to plants carrying the matching *R* genes, while they are virulent to plants without carrying the matching *R* genes (Xin-Zhong et al., 2002).

Race is defined by the avirulence gene that the pathogen lacks; therefore, it is able to overcome completely the resistance in the complementary host. This is based on the premise that a mutation at the *Avr* locus in the pathogen leads to nonrecognition by the host, hence leading to complete susceptibility. The race spectrum is defined by the combination of R genes it can overcome (Bus et al., 2011).

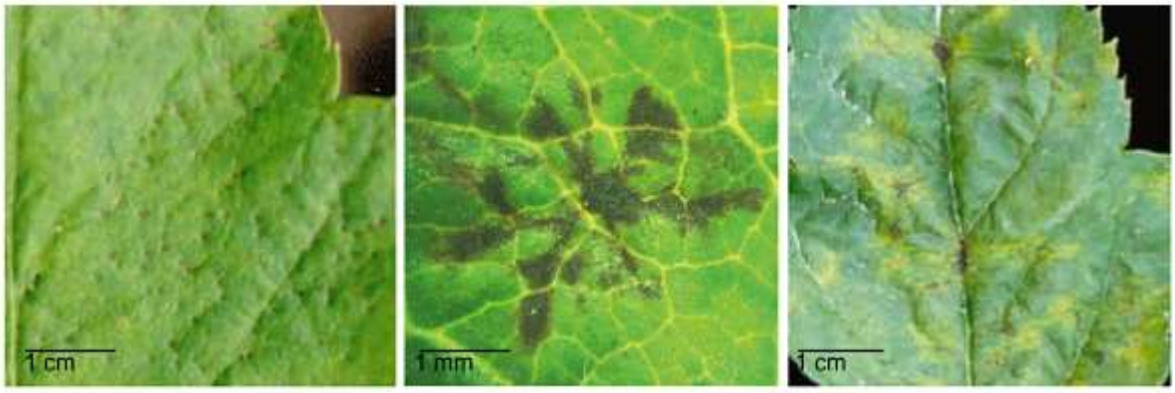

Figure 4. Characteristic scab resistance reactions on apple leaves: pin-point pit, hypersensitive response (left); stellate necrosis (middle); and chlorosis with limited sporulation (right) under glasshouse conditions (Bowen et al., 2011).

3.2.2. Major genes in apple governing Gene-for-Gene relationships with *V. inaequalis*

First proven resistance to apple scab was found in the small-fruited Asian *Malus* species: *M. floribunda* 821 *(Vf), M. pumila* R12740-7A *(Vr), M. baccata jackii (Vbj),* 'Hansen's baccata #2' *(Vb),* PI172623 *(Va), M. micromalus* 245-38 *(Vm),* and *M. atrosanguinea* 804 *(Vm).* The scab resistance genes of 'Golden Delicious' *(Vg),* GMAL 2473 *(Vr2),* 'Durello di Forli' *(Vd),* differential host 2 *(Vh2)* and host 4 *(Vh4)* (derived from *M. pumila* R12740-7A), and *M. sylvestris* W193B *(Vh8)* can be added if only one resistance source is incorporated into a new variety. However, it is not recommendable because the pathogen can overcome this resistance. A recent example is *Vf* resistance, and it has also happened with ephemeral resistance genes (Erdin et al., 2006).

Host breed specificity has long been demonstrated in isolates of *Malus* infected with *V. inaequalis.* To date, 18 gene-for-gene pairings have been identified between *V. inaequalis* races and *Malus* cultivars (Table 1). Only two scab *R* loci *(Rvi6* and *Rvi15)* in apple have been fully characterized (Deng et al., 2017). GfG relationships of importance for the present study are described below.

- Relationship (0): Host (0) can be defined as a host without the *R* gene; therefore, it is susceptible to all isolates of *V. inaequalis.* Nowadays, Gala cultivar is a commonly used universally susceptible host in many scab experiments and is widely used in many breeding programs around the world. It is also grown and used as parental in breeding programs. Due to its susceptibility, it has been chosen to represent h(0). The race (0) nomenclature means the nonpathogenic character to all hosts carrying the *R* gene and induces lesions only in hosts that do not carry known scab *R* genes (Bus et al., 2011).

- Relationship (1): Although *Rvi1* gene is overcome by an estimated 87% of the European pathogen population, both host (1) and race (1) have been extensively characterized, which makes this an important model system to better understand the basis of ephemeral, versus durable, *R* genes. The resistance is monogenetic and the *Rvi1* gene leads a necrosis-resistant response that exhibits weak sporulation (Bus et al., 2011).

- Relationship (3). Geneva is a red-leafed, open-pollinated selection of *M. pumila,* it was considered scab-resistant until 1951, when infection was reported in Nova Scotia. Geneva has been shown to have two resistance genes, tentatively designated *Vh3.1* and *Vh3.2,* which have been mapped to linkage group 4 (LG4). Resistance symptoms can range from hypersensitivity to chlorosis, but nonpathogenic isolates primarily exhibit necrosis responses. However, lesions from categories 2, 3, and 4 are seen under prolonged wet conditions (Bastiaanse et al., 2016; Patocchi et al., 2020).

Table 1. Nomenclature of the gene-for-gene relationships between *V. inaequalis* and *Malus* (Bus et al., 2011; Papp et al., 2020; Patocchi et al., 2020; Soriano et al., 2014).

Malus					*V. inaequalis*	
Differential host		Phenotype	Resistance locus			
Number	Accession		Historical	New	Avirulence locus	Race
h(0)	**Royal Gala**	**susceptibility**	**-**	**-**	**-**	**(0)**
h(1)	**Golden Delicious**	**necrosis**	***Vg***	***Rvi1***	***AvrRvi1***	**(1)**
h(2)	TSR34T15	stellate necrosis	*Vh2*	*Rvi2*	*AvrRvi2*	(2)
h(3)	**Geneva**	**stellate necrosis**	***Vh3***	***Rvi3***	***AvrRvi3***	**(3)**
h(4)	TSR33T239	hypersensitive response	*Vh4 = Vx = Vr1*	*Rvi4*	*AvrRvi4*	(4)
h(5)	9-AR2T196 and **OR45t132 (syn. PRI 333-9)**	**hypersensitive response**	***Vm***	***Rvi5***	***AvrRvi5***	**(5)**
h(6)	Priscilla	chlorosis	*Vf*	*Rvi6*	*AvrRvi6*	(6)
h(7)	Malus x floribunda 821	hypersensitive response	*Vfh*	*Rvi7*	*AvrRvi7*	(7)
h(8)	B45	stellate necrosis	*Vh8*	*Rvi8*	*AvrRvi8*	(8)
h(9)	K2	stellate necrosis	*Vdg*	*Rvi9*	*AvrRvi9*	(9)
h(10)	A723–6	hypersensitive response	*Va*	*Rvi10*	*AvrRvi10*	(10)
h(11)	A722–7	stellate necrosis/chlorosis	*Vbj*	*Rvi11*	*AvrRvi11*	(11)
h(12)	Hansen's baccata #2	chlorosis	*Vb*	*Rvi12*	*AvrRvi12*	(12)
h(13)	Durello di Forl`ı	stellate necrosis	*Vd*	*Rvi13*	*AvrRvi13*	(13)
h(14)	Dulmener Rosenapfel	chlorosis	*Vdr1*	*Rvi14*	*AvrRvi14*	(14)
h(15)	GMAL2473	hypersensitive response	*Vr2*	*Rvi15*	*AvrRvi15*	(15)
h(16)	MIS op 93.051 G07–098	hypersensitive response	*Vmis*	*Rvi16*	*AvrRvi16*	(16)
h(17)	Antonovka APF22	chlorosis	*Va1*	*Rvi17*	*AvrRvi17*	(17)
h(18)	Selection 1980-015-025	hypersensitive response	*Vd3*	*Rvi18*	*AvrRvi18*	(18)
h(19)	Honeycrisp	Ongoing studies	-	*Rvi19*	*AvrRvi19*	(19)
h(20)	Honeycrisp	Ongoing studies	-	*Rvi20*	*AvrRvi20*	(20)

*Bold rows show the cultivars used in the present work.

- Relationship (5). *Malus micromalus* 245-38 and *Malus x atrosanguinea* 804 are considered highly resistant sources conditioning hypersensitivity responses, visible within three days after inoculation. Both accessions also carried a chlorosis conditioning gene for scab resistance. Genetic markers for *Rvi5* were identified in *M. x atrosanguinea* 804 derivatives NY748828-12 and OR45t132 (Bus et al., 2011).

3.3. Management of apple scab

The most effective scab management begins with resistant cultivars (Ward, 2012). However, several strategies are needed to control apple scab on the long run, including adequate cultural practices, and chemical control.

3.3.1. Resistant cultivars

The first attempts to develop scab-resistant varieties were made in Germany and the US (Ignatov and Bodishevskaya, 2011; Peil et al., 2010). In Germany, Schmidt (1938) aimed to combine resistance found in several *Malus* species with fruit size and quality traits. Then, the work was continued with cultivars carrying polygenic scab resistance e.g., 'Antonovka' and 'Ernst Bosch' (Schmidt, 1938).

In 1945, there was a contribution between three American universities: Purdue University, Rutgers University, and University of Illinois. It was called PRI Apple Breeding Program in which several researchers determined scab inheritance in a large germplasm of *Malus* species, develop and screen the resulting hybrid progenies. Up to 2006 1.500 apple selections were outstanding from the PRI program, of which 44 were selected for further testing, and 16 of them were named as cultivars (Peil et al., 2010). Nowadays as breeders have access to more and better tools, new cultivars with various effective resistances can be released at shorter intervals by using DNA technologies (Gessler and Pertot, 2012).

3.3.1.1. Plant breeding for apple scab resistance

Breeding resistant apple cultivars represent a long-term solution for commercial plantations. One of the tools for breeding is marker-assisted selection (MAS) that involves tracking the inheritance of resistance genes by using molecular markers. It is usually validated for genes segregated by Mendelian laws such as apple scab resistance genes e.g., *Rvi6 (Vf)*, which is inherited in a dominant state (Laurens et al., 2018; Zelmene et al., 2022).

Nowadays *Rvi6* resistance gene, which was initially identified in the selection '*Malus floribunda 821*' during the PRI program, is the basis for the majority of contemporary apple scab-resistant cultivars. Additionally, the organic apple industry continues to rely on 'Golden Delicious' *(Rvi1)* and other susceptible cultivars. For instance, in countries like Switzerland and Germany, where integrated and organic production systems are well established, scab-resistant apples account for no more than 5–6% of the market, and in Italy scab-resistant apples account for less than 1% (Ignatov and Bodishevskaya, 2011).

Up to date twenty different *Rvi* resistance genes have been identified in apple and at least one molecular marker has been developed for each gene. Also, there are several programs working in database for marker assisted breeding, pyramiding multiple apple scab resistance genes, and quantitative trait loci (QTL) associated with apple scab. Gene pyramiding seems promising for breeders to ensure that new cultivars have at least two or more resistance genes to establish long-term apple scab resistance (Zelmene et al., 2022).

3.3.1.2. Recombinant DNA technology

The use of recombinant DNA technology to transform a scab-susceptible cultivar into a scab-resistant cultivar is a promising idea, since all the characteristics of a cultivar are conserved in this type of genetic manipulation, unlike in the case of classic breeding. Foreign genes from a wide variety of sources have been used to transform and introduce resistance into popular susceptible cultivars, and some of these efforts have even reached the stage of field trials (Gessler and Pertot, 2012).

In the US, 106 field trials have been notified in the database of the United States Department of Agriculture (USDA) since 1991, and the number of applications has remained constant over the last 30 years. On the other hand, the EU registered only eight notifications and only two of them were published after 2011 (Hanke and Flachowsky, 2010). In 2021 a field trial was carried out in the Netherlands to evaluate apple plants of 'Gala' expressing the HcfVF2 *(Rvi6)* scab resistance gene (Schlathölter et al., 2021). This limited amount of apple trials in the EU might be due to the existing GMO regulations and the lack of acceptance in parts of the population (Schröpfer et al., 2022).

3.3.2. Chemical control

Fungicides are currently the main control for apple scab. Intensive pesticide application is required in order to stop the spread of apple scab and produce commercially acceptable fruits, with up to 20 sprayings every growing season (Zelmene et al., 2022).

Carisse (2006) indicated that spray programs include protectant fungicides with multisite activity that affect primarily spore germination, such as dithianon (quinone class), captan (phthalimide class) and Bordeaux mixture (copper-based), or systemic fungicides which are absorbed by the plant and affect fungal growth, such as dodine (guanidine class), difenoconazole (demethylation inhibitor group; DMIs), pyraclostrobin (quinone-outside inhibitors; QoIs) and the anilinopyrimidine cyprodinil (Carisse et al., 2006).

However, main problems of such intensive chemical control include the negative impacts on the environment, workers and consumers health, and they are also expensive. Furthermore, systemic fungicides used in curative applications are known for their significant risk of developing resistance, and some have shown to lose efficacy over time e.g., dodine and Quinone outer binding site of the cytochrome bc1 complex Inhibitor – QoIs (Fontaine et al., 2009; Mondino et al., 2015).

The intrinsic property of each pathogen to develop resistance is known as inherent risk and *V. inaequalis* is classified as a pathogen with a high inherent risk of generating resistance (Carbone, 2017). According to Brent and Hollomon (2007), the most important factors that determine this feature are:

- Pathogen producing many generations per season. The shorter the time between each generation, the more frequently it is necessary to apply fungicide which leads to an increase in selection pressure in favor of resistant individuals.
- Abundance of sporulation that leads to high variability of genomes in which mutations and selection can occur. Therefore, it is more likely the spreading of resistant mutant individuals.
- Ability to spread spores between plants, crops and regions.
- Ability to infect at different stages of the crop, which determines the need for chemical treatments at different times (Brent and Hollomon, 2007).

Nowadays, using less fungicides for plant disease control is highly preferred to minimize the negative effects of intensive spraying. Therefore, integrated diseases strategies for apple scab might include a combination of fungicides and systemic resistance-inducing products (Chatzidimopoulos et al., 2020). Furthermore, developing simulation models based on Mills' periods can be effective for making decision on targeted applications of fungicides against primary infections. Most part of the models include duration of leaf wetness and ascospore maturation to assess the risk of scab infection, but new components can be added such as susceptible leaf area (Beresford et al., 2004; Mills and LaPlante, 1951).

3.3.3. Cultural practices

One of the major challenges of the twenty-first century is to provide high quality crops for its increasing population. In order to meet this demand, farmers must produce more crops in line with the market requirements. Despite the importance of chemical control in the food system, its persistent overuse has caused a number of adverse effects. Thus, several strategies should be applied to provide high-quality fresh products to future generations taking into consideration economic viability and ecological sustainability (Kaur and Kaur, 2020).

Therefore, cultural practices are crucial to long-term orchard establishment, including improving air circulation by pruning trees to facilitate faster fruit and leaf drying, decreasing the fungal inoculum in the next spring by destroying fallen leaves and fruit. Some commercial orchards mow fallen leaves and apply nitrogen in order to stimulate leaf tissue decomposition. Fungicide effectiveness is also reduced when these practices are not implemented. Also, to avoid additional stress, it is recommendable to provide water and mulch if trees are defoliated early in the year and fertilize in the fall based on the soil needs (Ward, 2012).

4. MATERIALS AND METHODS

4.1. Fungal material

V. inaequalis isolates used for this study were previously obtained from four scab race indicator cultivars: 'Golden Delicious' (carrying the resistance gene *Rvi1*), 'Geneva' (*Rvi3,* complex), sel. OR45t132 (*Rvi5*), and 'Gala' (no *R*-gene) collected in Soroksár orchard (Budapest, Hungary). These isolates were maintained by successive culturing on Potato Dextrose Agar (PDA) plates **(Table 2).**

Table 2. Number of *V. inaequalis* racial isolates used in the study, and their nomenclature (Bus et al., 2011).

Commercial cultivar	Race indicator	Pathogen race	Nr. of isolates
Gala	-	race 0	27
Golden Delicious	*Rvi1*	race 1	12
Geneva	*Rvi3,* complex	race 3	12
OR45t132	*Rvi5*	race 5	19
Total			70

In order to evaluate the fitness costs of *V. inaequalis* races, the following experiments were carried out at the laboratory: (1) morphological characterization of isolates, (2) mycelium growth under different conditions, (3) germination rate from conidia spores, (4) detached leaf assay, and (5) imaging of microscopical structures. During the experiments samples that showed contamination were excluded from the analyses, resulting in smaller size.

4.2. Morphological characterization of isolates

Cultural features based on variability of isolates were evaluated on *V. inaequalis* isolates (n = 60), based on the work described by Nasonov (2020). Three characteristics were analyzed on four weeks old isolates, including: color (RAL color chart, https://www.ralcolorchart.com/ral-colors), density (underdeveloped, loose, and dense mycelium, **Figure 5**), and texture (felt, velvety, woolly, and fluffy, **Figure 6**) of the main air mycelium.

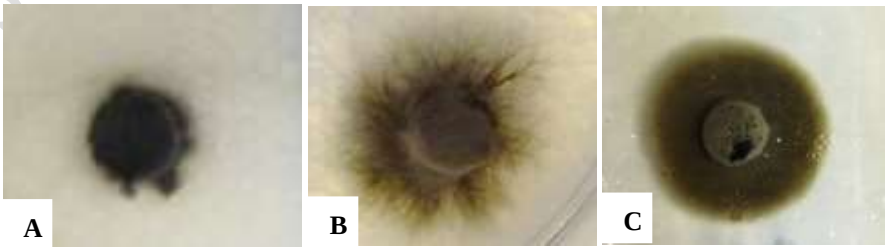

Figure 5. Morphotypes of apple scab pathogen isolates according to the density of aerial mycelium. **A.** Underdeveloped, **B.** Loose, **C.** Dense.

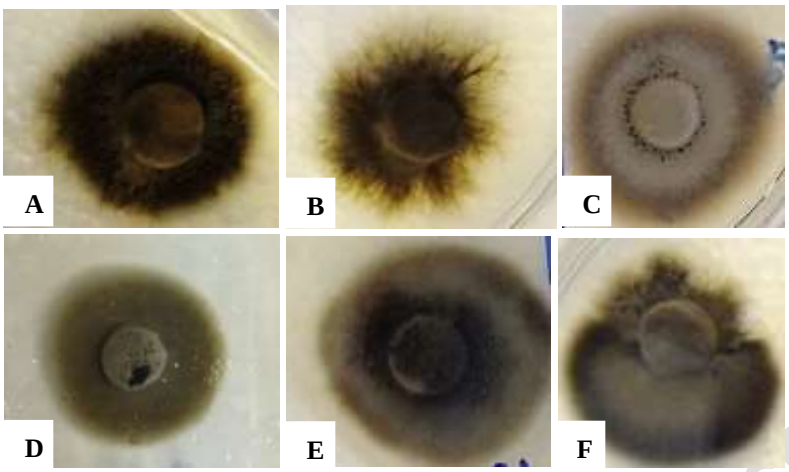

Figure 6. Morphotypes of apple scab pathogen isolates according to the texture of aerial mycelium. **A.** Spongy, **B.** Cobwebbed, **C.** Felt, **D.** Velvety, **E.** Woolly, **F.** Fluffy.

4.3. Mycelium growth of *V. inaequalis* isolates

PDA culture medium at 4% was sterilized in an autoclave (Sanyo Electric Co., Ltd., Japan) for 15 min at 120ºC, and poured to disposable Petri-dishes. Under laminar flow hood (LN 120, Nüve A.S, Turkey), two perpendicular lines were drawn at the bottom of each Petri dish in order to have four quadrants. A mycelium plug of 6 mm diameter was obtained for each isolate (from the microorganism repository of the Fruit Department) by using a sterile microbiological cork borer. Subsequently, the sample was placed at the center of the quadrant on the surface of PDA medium.

Following the protocol mentioned before, three experiments were established in order to evaluate the mycelium growth under different conditions: Treatment 1 (T1), Treatment 2 (T2), and Treatment 3 (T3) **(Table 3).** Group T1 was grown at an optimal temperature of 19°C. Groups T2 and T3 at 14ºC and 19ºC, respectively. Additionally, group T3 was applied H_2O_2 1 mM as stress solution. The H_2O_2 was prepared from a stock solution H_2O_2 at 3%. Three biological replicates were prepared in all cases.

Table 3. Experiments to evaluate the mycelium growth of *V. inaequalis* isolates.

Experimental group	Label	T (°C)	Stress solution
Treatment 1	T1	19	-
Treatment 2	T2	14	-
Treatment 3	T3	19	H_2O_2

Petri dishes were sealed with parafilm tape and incubated under the same light conditions during 28 days in an incubator (FTC 90E, VELP Scientifica, Inc. Italy). After that period, measures of each isolate were taken: width, length, and diagonal diameter by using a caliper **(Figure 7).**

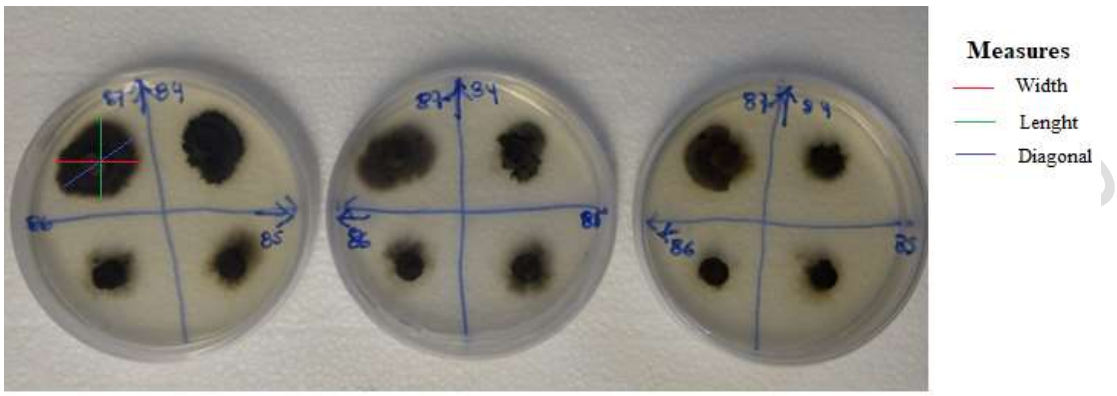

Figure 7. Evaluation of mycelium growth of *V. inaequalis* isolates.

4.4. Cellophane experiment: Conidial production

To prepare cellophane cultures, membranes were cut into 8 cm disks and soaked in water during 12 hours. Soaked disks were layered between filter paper and sterilized in autoclave for 15 min at 120ºC. Under laminar flow hood (LN 120, Nüve A.S, Turkey) a mycelium sample of 6 mm diameter was obtained from a previous repository (four weeks old isolates) by using a sterile microbiological cork borer. The sample was placed in an Eppendorf tube with 0.5 ml sterile water. Then, it was grinded with a pellet pestle until obtaining a homogenic suspension of blended mycelium which was transferred to the cellophane-covered PDA surface and distributed throughout the cellophane disk. Plates were sealed with parafilm and incubated at 19ºC under UV blacklight (24 Watt, 390 nm) for 12 hours per day **(Figure 8A).**

At day 7 of incubation, cellophane membranes were removed **(Figure 8B),** cut into small pieces and placed into 50 ml of water in an Erlenmeyer flask **(Figure 8C).** They were put on an orbital shaker (Universal Shaker SM 30 A, Manufacturer Edmund Bühler, Germany) for 40 minutes at 150 rpm to remove the conidia from the cellophane membrane **(Figure 8D).**

The conidial suspensions were transferred to new flasks by using a double layer of gauze as a filter to clean the suspension of any mycelium and other particles. Then, the spore concentration of the suspensions was assessed at 20x with the light microscope Olympus BX41 (Olympus Corporation, Japan) using a hemocytometer **(Figure 8E).** Results were also analyzed on Days 10, 13, 17, 20, and 27 to keep track of the conidia production, and were reported as conidia/ml.

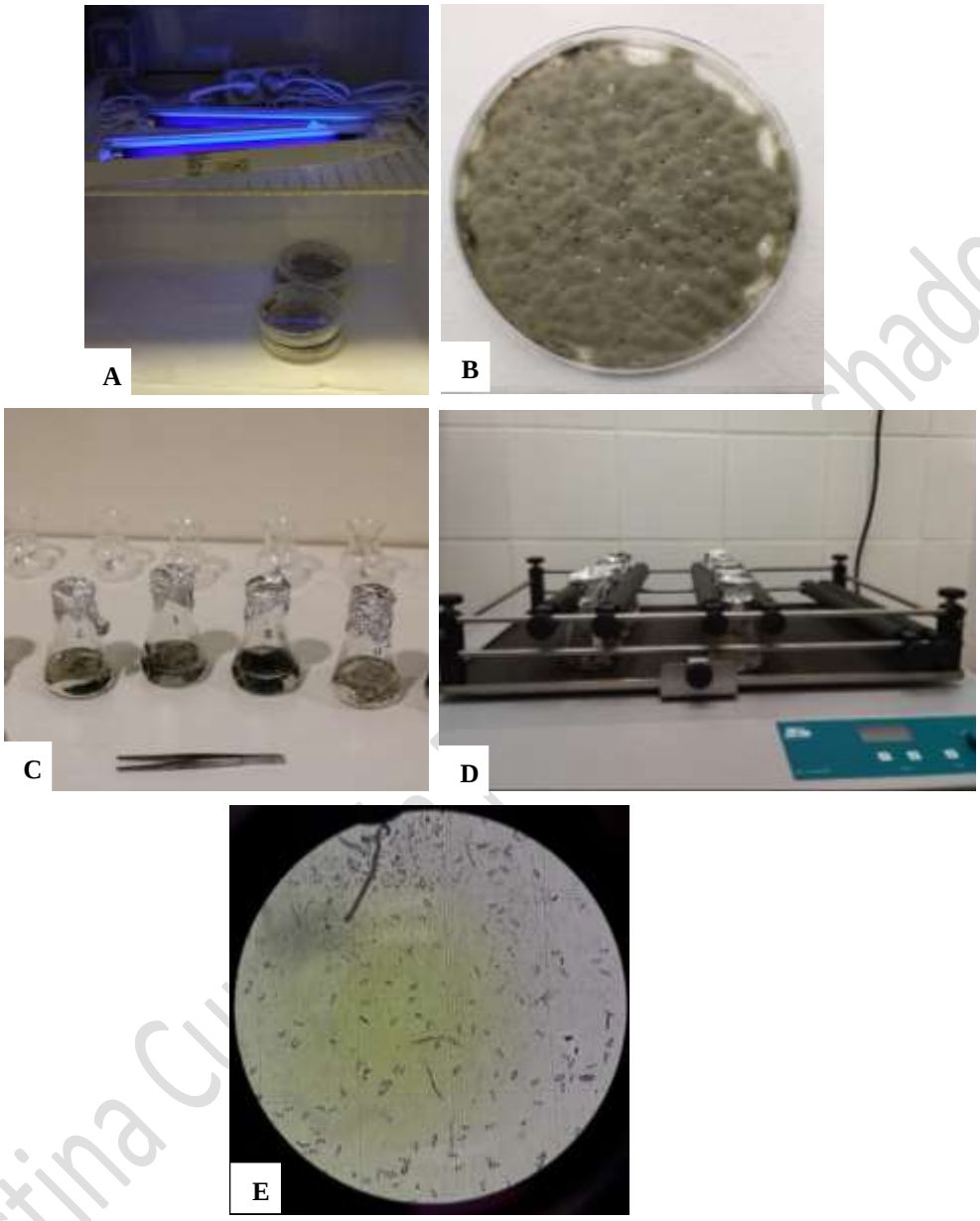

Figure 8. *V. inaequalis* conidia spore obtention. **A.** Incubation of plates at 19ºC in UV light. **B.** Cellophane membrane with conidia sporulation after 7 days of incubation. **C.** Conidial suspension on an orbital shaker. **D.** Conidia suspension after filtration through gauze. **E.** Spore counting by using hemocytometer.

As an alternative to cellophane cultures, conidia suspensions were also obtained by harvesting conidia directly from naturally occurring scab lesions. Eight infected leaves were collected randomly from each of the four investigated cultivars in June 2022. Eight leaf discs (one per leaf per cultivar) with one sporulating lesion on each disc were cut using a 6 mm wide cork borer. To suspend the conidia, the discs were placed into an Eppendorf, and after adding 500 µl of tap water, the samples were vortexed for 5 min. The spore concentration of the suspensions

was set to approx. 5.6 x10^4 conidia/ml by using a hemocytometer. The viability of the conidia was checked by dropping 20 µl of suspension to a 2% water agar plate and counting the number of germinated conidia/100 conidia after 24 hours of incubation at room temperature.

4.5. Pathogenicity testing

4.5.1. Detached leaf assay - DLA

Young undamaged leaf samples of the four studied cultivars were collected in June 2022 in Soroksár Orchard (Budapest, Hungary). Carefully, they were immersed in water until reaching the laboratory facilities. Leaves were washed softly for 15 minutes with a continuous flow of water, and a small section of petiole was chopped off to eliminate the surrounding dead tissue. Then, leaves were dried out with paper towel and placed on 2% agar water culture medium in such a manner that the petiole was partially immersed in the medium **(Figure 9).**

Figure 9. Detached-leaf assay in apple leaves.

Plates were prepared in two repetitions for the 16 combination (4 cultivars x 4 isolates). A volume of 5 µL of the new conidia suspension was placed on four different spots on the leave surface, and the plates were sealed with parafilm and placed to 19 ºC with constant light. After 4 days the leaves were washed again with tap water and dried under laminar flow. Symptoms were analyzed 14 days post inoculation following the protocol described by Chevalier et al. (1991). **Table 4** shows the scale of scab symptoms on the leaves and the response category.

Table 4. Evaluation of scab symptoms and response categories (Chevalier et al., 1991).

Rank	Symptoms	Response category
0	None	-
1	Pin point pits	Resistant
2	Chlorotic lesions	Resistant
3a	Necrotic and some chlorotic lesions, very weak sporulation	Moderately resistant
3b	Clearly sporulating chlorotic and necrotic lesions	Moderately susceptible
4	Abundantly sporulating lesions covering most of the leaf area	Susceptible

4.5.2. Germination rate

The viability of the conidia was checked using the suspension of the previous experiment. Spore concentration was set to approx. 5.6 $x10^4$ conidia/ml by using a hemocytometer. 20 µl of suspension was dropped on 2% water agar plate. The number of germinated conidia/100 conidia was counting after 24 hours of incubation at room temperature by using a light microscope. According to the protocol described by Aylor and Sanogo (1997) (Aylor and Sanogo, 1997), a conidium was considered germinated if the length of the germ tube was greater than or equal to the length of the conidium (~20 µm).

4.5.3. *V. inaequalis* and *Malus domestica* microscopical structures

A small sample of mycelium was taken from the previous repository of isolates and placed on the slide by using an inoculation loop. A volume of 10 µL of methyl red solution dye was dropped on the sample to increase the contrast of the mycelium structures, then a cover slide was placed on. Measures of each isolate were taken including width and length of the mycelium structures in random fields at 40x with a light microscope. Moreover, infection on leaves was evaluated by microscope to identify characteristic structures (E.g., stroma structures, running hyphae, and conidia spores) from the interaction between *V. inaequalis* and apple at 40x with a light microscope.

4.6. Statistical methods

Morphological features of *V. inaequalis* isolates from four scab race indicator cultivars ('Gala', 'Golden Delicious', and 'Geneva', and sel. OR45t132) were evaluated by using descriptive statistics to have proportions for the studied variables. Chi-Square test was used to determine significant correlation between two categorical variables (air mycelium color, texture, and density). Results were reported with test value, df and p (Chi2 (df)= X-square; p).

Welch ANOVA model was applied to assess the differences of mycelium growth (mm) between *V. inaequalis* isolates from four scab race indicator cultivars. The test was performed for each treatment (19ºC, 14ºC, H_2O_2). Before using the variable mycelium growth, average was calculating between width, length, and diagonal diameter to have a unique measure.

Welch ANOVA model had the assumptions of normally distributed residuals and non-homogeneity of variances, besides independent samples. Normality was checked with Kolmogorov-Smirnov test and also graphically by histogram and QQ-plot, while homogeneity of variances was tested by using Levene's test. Pairwise comparisons were calculated by Games-Howell post hoc test (for not equal variances). All used statistical tests were two-sided, and the significance level was set at α=0.05. Additionally, Pearson correlation coefficient (R) was calculated to measure the linear correlation between the variables "mycelium growth" and "spore concentration".

For the DLA experiment, proportions for symptoms and response categories were obtained. Furthermore, three observations were made per suspension to evaluate conidial production over time. Welch ANOVA model was applied to evaluate the differences of conidia/ml over time between *V. inaequalis* isolates from four scab race indicator cultivars. This model had the assumptions of normally distributed residuals and non-homogeneity of variances, besides independent samples.

Normality was checked with Kolmogorov-Smirnov test, d'Agostino test, and graphically by histogram and QQ-plot. Homogeneity of variances was tested by using Levene's and Bartlett test. Pairwise comparisons were calculated by Games-Howell post hoc test (for not equal variances). All used statistical tests were two-sided, and the significance level was set at $\alpha=0.05$.

Statistical analyses were performed by using the statistical software package R (Version 4.4.1. R Foundation for Statistical Computing, Vienna, Austria).

5. RESULTS

In order to evaluate the fitness costs of *V. inaequalis* races, the following experiments were carried out at the laboratory: morphological characterization of isolates, mycelium growth under different conditions, conidia production, and pathogenicity testing by DLA.

5.1. Morphological characterization of isolates

V. inaequalis isolates were previously obtained from four scab race indicator cultivars: 'Gala' (no R-gene), 'Golden Delicious' *(Rvi1),* and 'Geneva' *(Rvi3,* complex), and sel. OR45t132 *(Rvi5).*

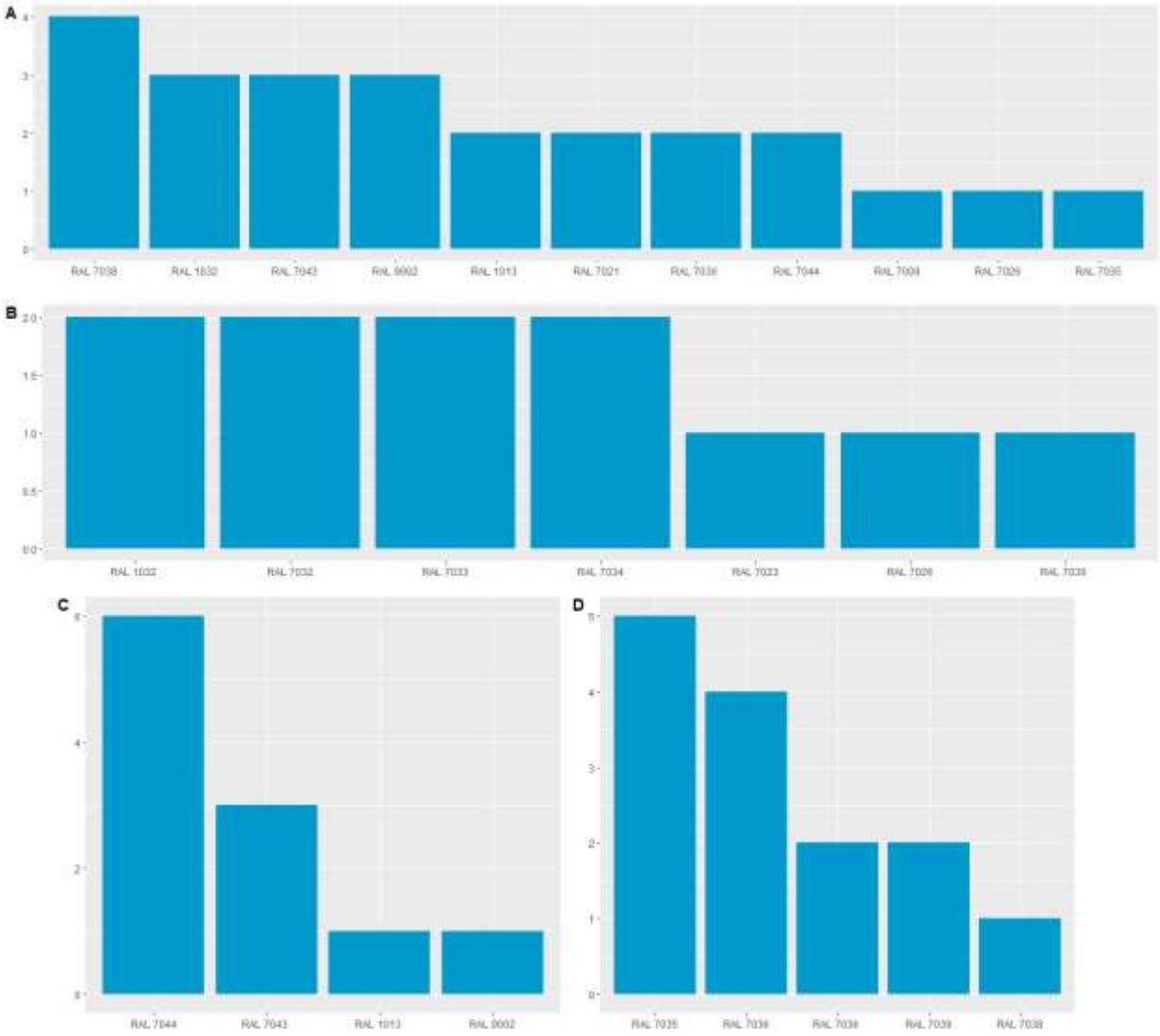

Figure 10. Scale color frequency of aerial mycelium on *V. inaequalis* isolates. **A.** 'Gala', **B.** 'Geneva', **C.** 'Golden Delicious', and **D.** OR45T132.

Cultural features based on variability of isolates were evaluated on sixty *V. inaequalis* isolates. Ten isolates were excluded from these analyses due to contamination. Based on the work described by Nasonov (2020), three characteristics were analyzed including: color (available color scales), density (underdeveloped, loose, and dense mycelium), and texture (felt texture, velvety, woolly, and fluffy) of the main air mycelium.

Isolates from 'Gala' **(Figure 10A)** showed more color diversity in comparison with those from 'Geneva' **(Figure 10B),** 'Golden Delicious' **(Figure 10C),** and OR45t132 **(Figure 10D).** The predominant color for 'Gala' was identified as agate grey (RAL 7038), and 17% of the samples presented it. The main colors for 'Geneva' were broom yellow (RAL 1032), pebble grey (RAL 7032), cement grey (RAL 7033), and yellow grey (RAL 7034) with 18% each. 55% of the isolates were silk grey (RAL 7044) for 'Golden Delicious', and 43% were platinum grey (RAL 7036) for OR45T132. The Chi-Square test indicated that the color is not independent from the isolates from different host of origin Chi2 (57) = 134.95; p<0.005.

In terms of density, 'Gala' and 'Geneva' showed mostly dense mycelium growth, 79% **(Figure 11A)** and 73% **(Figure 11B),** respectively. On the other hand, 'Golden Delicious' **(Figure 11C)** and OR45T132 **(Figure 11D)** showed loose mycelium growth, 64% each. The Chi-Square test indicated that the density is not independent from the isolates from different host of origin Chi2 (6)= 19.33; p<0.005.

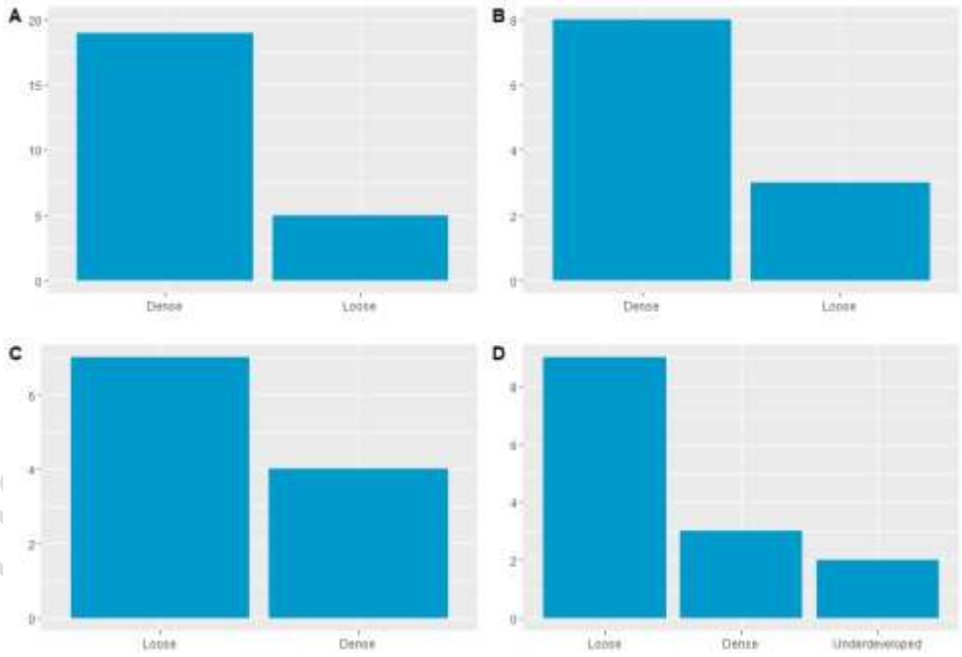

Figure 11. Density frequency of aerial mycelium on *V. inaequalis* isolates. **A.** 'Gala', **B.** 'Geneva', **C.** 'Golden Delicious', and **D.** OR45t132.

For texture, 'Gala' isolates presented mostly velvety texture (33%, **Figure 12A**). 'Geneva' presented velvety (18%) and cobwebbed (18%) textures **(Figure 12B)**. 'Golden Delicious' and OR45T132 showed cobwebbed

texture, 45% **(Figure 12C)** and 71% **(Figure 12D)**, respectively. The Chi-Square test indicated that the texture is not independent from the isolates from different host of origin Chi2 (15)= 27.32; p<0.05.

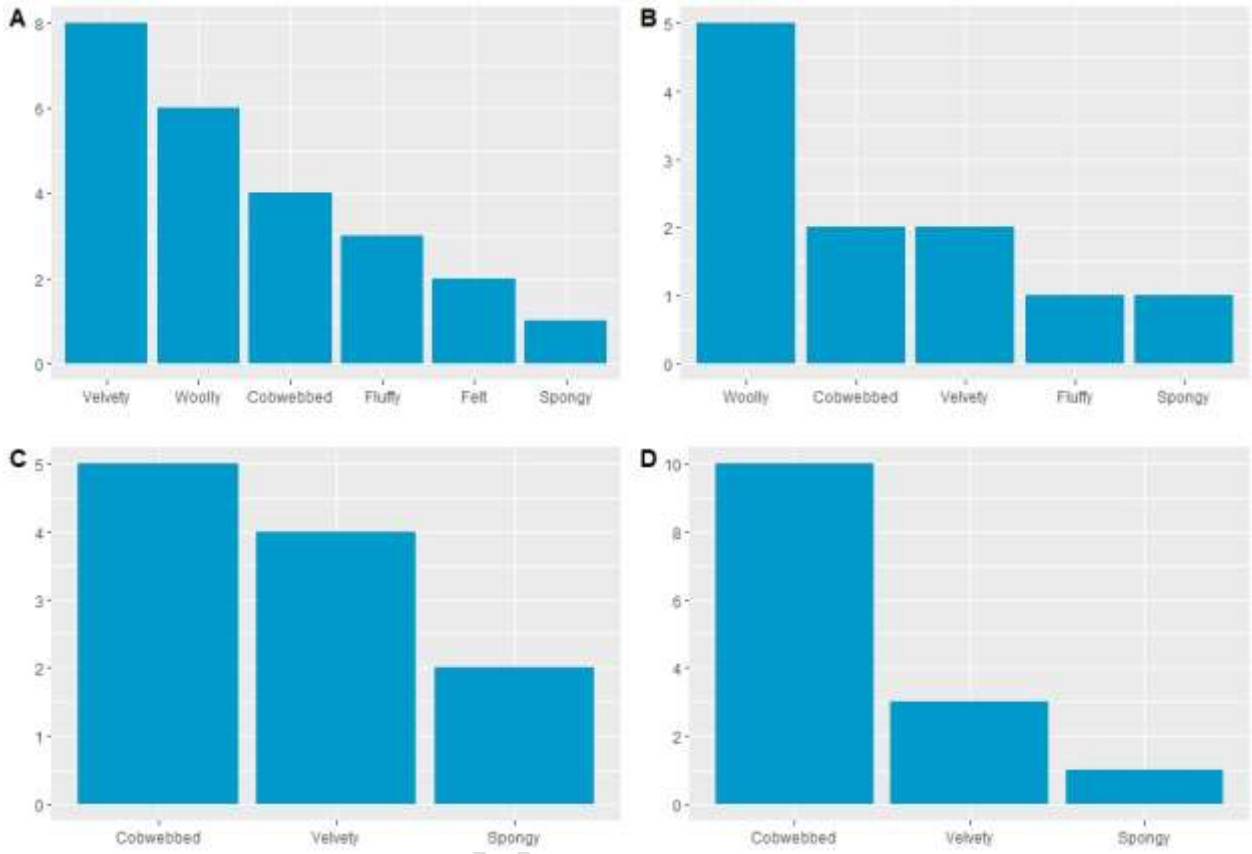

Figure 12. Texture frequency of aerial mycelium on *V. inaequalis* isolates. **A**. 'Gala', **B.** 'Geneva', **C.** 'Golden Delicious', and **D.** OR45t132.

5.2. Mycelium growth of *V. inaequalis* isolates

Welch ANOVA tests were applied individually for each treatment (T1, T2, and T3) in order to assess the differences of mycelium growth (mm) between *V. inaequalis* isolates from four scab race indicator cultivars. In case of T1 (19ºC), the Welch ANOVA test provided significant difference in average growth between the investigated isolates from different host ($p<0.0001$). Then, Games-Howell post hoc test (for not equal variances) showed that 'Gala' and OR45t132 groups differed from each other significantly ($p < 0.001$) in average growth (mm), being 'Gala' larger than OR45t132 isolates. The other pairs were not significantly different.

This pattern was repetitive for treatments T2 – 14ºC ($p < 0.001$) and T3 – H_2O_2 ($p < 0.001$), in which in 'Gala' presented statistically higher average growth (mm) compared to ORT45t142 **(Figure 13).** Additionally, Pearson correlation coefficient ($R = 0.89$) showed strong correlation between the variables 'mycelium growth' and 'spore concentration' which is described below.

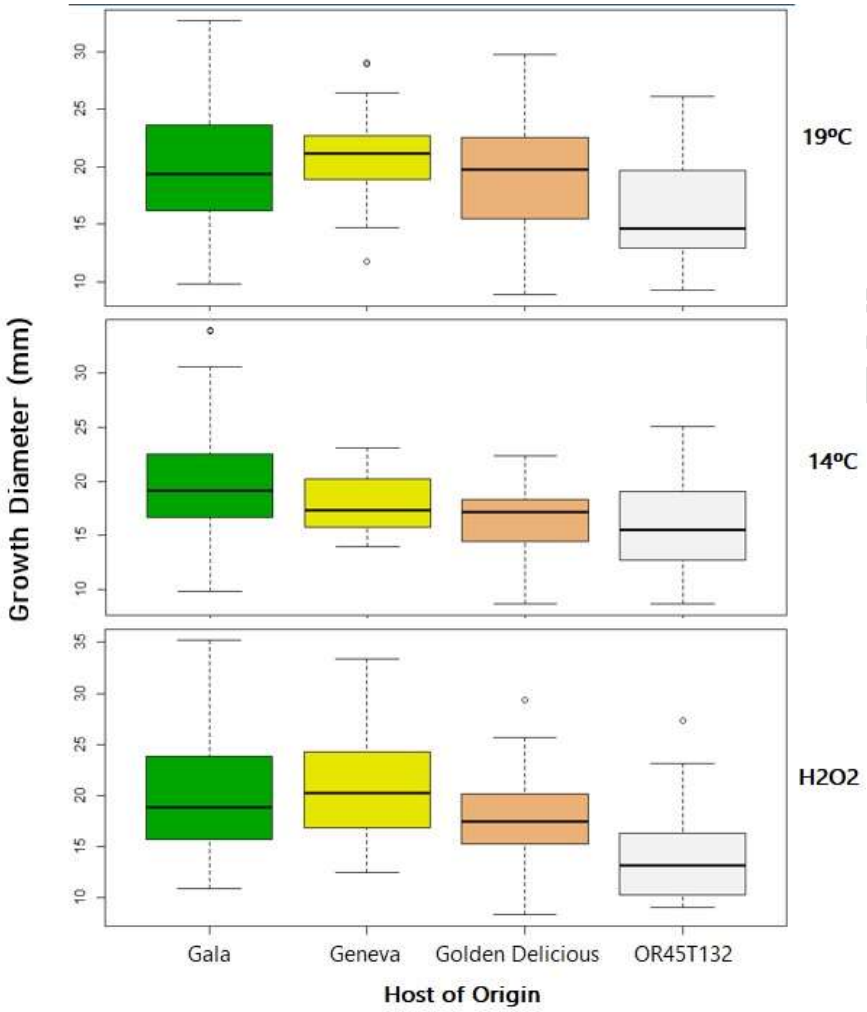

Figure 13. Mycelium growth of *V. inaequalis* isolates under different conditions: T1 (19ºC), T2 (14ºC), and T3 (H_2O_2).

5.3. Cellophane experiment: Conidial production

At first sight, conidia suspensions provided brief insight of the difference in spore concentration between groups. **Figure 14A** shows the difference of colors between 'Gala', 'Geneva', and OR45t132 suspensions, where it appears that 'Gala' has the highest spore density followed by 'Geneva', and then OR45t132. This visual assumption were confirmed by using Welch ANOVA and Games-Howell post hoc test, which showed that 'Gala' **(Figure 14B)** reached the highest spore density yields compared to 'Geneva' **(Figure 14C),** and OR45t132 (**Figure 14D,** $p < 0.001$). 'Golden Delicious' was excluded from these analyses due to failure to sporulate regardless several attempts with different isolates. Furthermore, it was seen in the suspensions that conidia spores presented typical shape, however, in the case of 'Geneva' they seemed particularly elongated **(Figure 15C).**

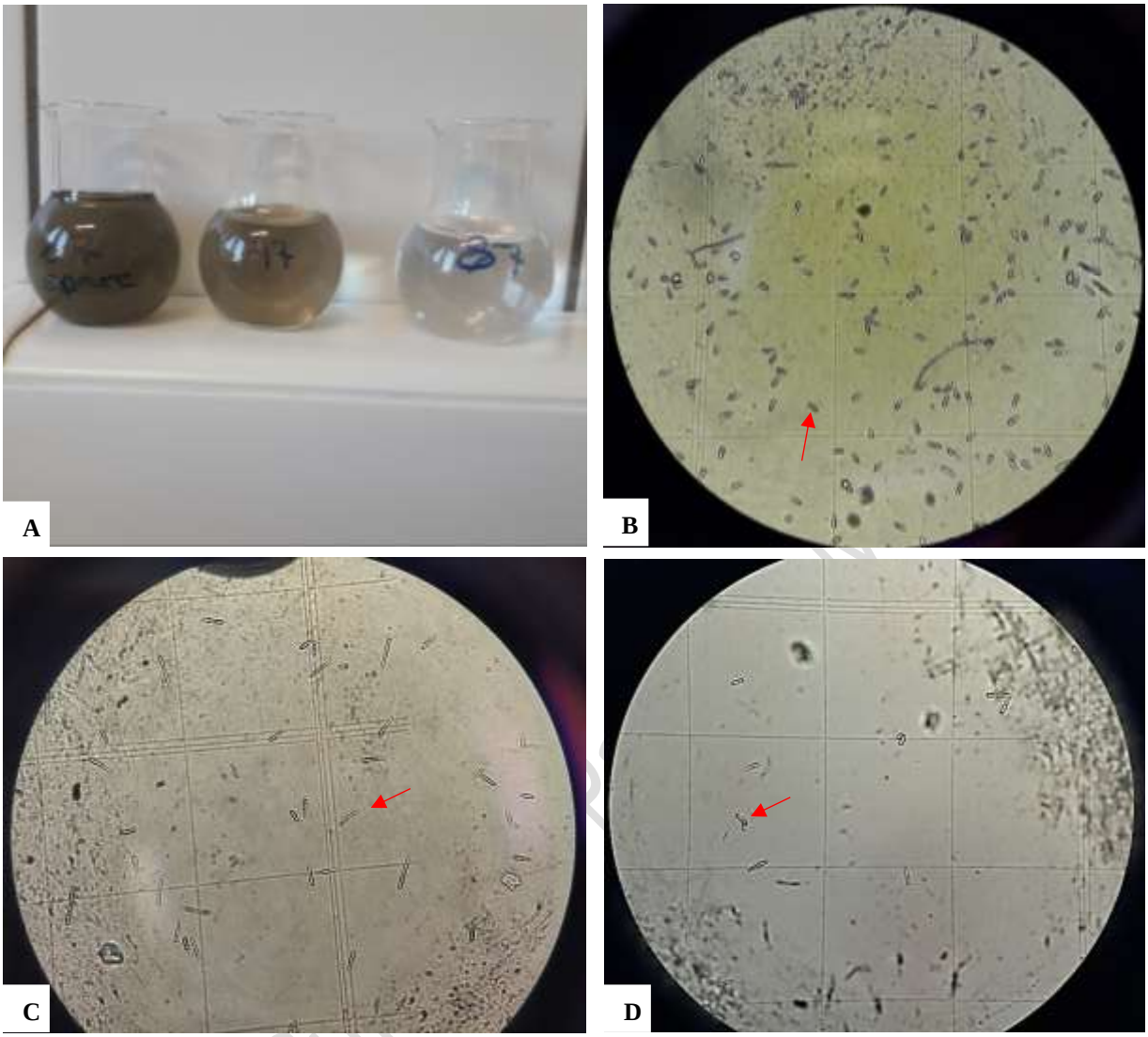

Figure 14. A. Conidial suspension obtained from 'Gala', 'Geneva', and OR45t132 isolates (left to right). Conidia spores from **B.** 'Gala', **C**. 'Geneva', and **D.** OR45t132 suspensions on Day 7.

A time course tracking revealed that for 'Gala', a first maximum conidia production on cellophane covered PDA plates was reached on Day 17 **(Figure 15).** A sharp decline was observed after it, followed by a new increase in yields on Day 27. The dual peak cycle of conidia production is explained by the germination of the first crop of conidia, followed by a second cycle of mycelial development and renewed sporulation.

Optimal yields for 'Gala' seen on Day 17 and 27 were 8.98 x 10^4 and 8.95 x 10^4 conidia/ml, respectively. Lowest yield was observed on Day 10 (1.05 x 10^4 conidia/ml, **Table 5**).

Table 5. Time course of light-induced conidia production (conidia/ml) by mycelium of *V. inaequalis* on cellophane-covered PDA agar.

Day	7	10	13	17	20	27
'Gala'	1.46×10^4	1.05×10^4	6.66×10^4	**8.98×10^4**	6.46×10^4	**8.95×10^4**
'Geneva'	**9.33×10^3**	6.83×10^3	**7.00×10^3**	3.00×10^3	6.33×10^3	3.33×10^3
OR45t132	3.66×10^3	**1.86×10^4**	**6.00×10^3**	3.37×10^3	2.36×10^3	1.0×10^3

'Geneva' had a first maximum conidia production on cellophane covered PDA plates on Day 7 (9.33×10^3conidia/ml, **Figure 15**). A slight decline was observed after it, followed by a new increase in yields on Day 13 (7.00×10^3 conidia/ml). Lowest yield was observed on Day 17 (3.00×10^3 conidia/ml, **Table 5**).

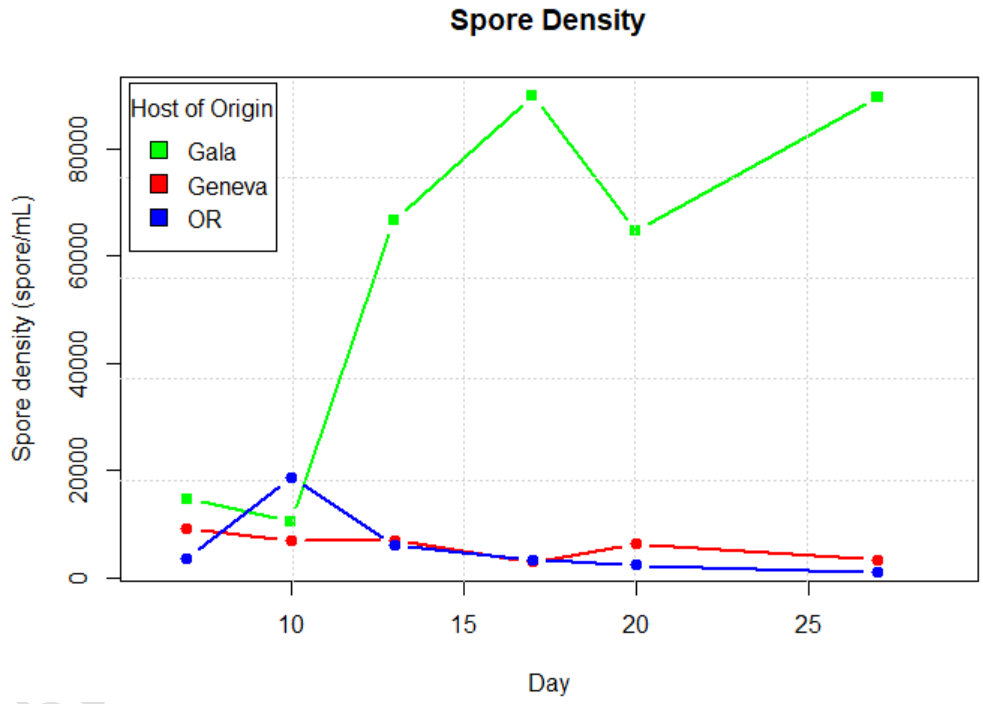

Figure 15. Spore density of *V. inaequalis* isolates from four scab race indicator cultivars ('Gala', 'Golden Delicious', and 'Geneva', and sel. OR45t132) on Day 7,10, 13, 17, 20, and 27.

On the other hand, OR45t132 had the maximum conidia production peaks on Day 10 (1.86×10^4 conidia/ml, **Figure 15**) and Day 13 (6.00×10^3 conidia/ml). Lowest yield was observed on Day 27 (1.0×10^3 conidia/ml, **Table 5**).

5.4. Pathogenicity Testing by Detached Leaf Assay – DLA

Four conidia suspensions were obtained directly from naturally occurring scab lesions in leaves of 'Gala', 'Geneva', 'Golden Delicious', and OR45t132, and then, detached leaves of the same four cultivars were inoculated with each suspension. All conidia suspensions caused obvious scab symptoms on their original host cultivars **(Table 6).**

Table 6. Percentage of successful infection in 16 isolate/host combinations by drop inoculating detached leaves.

	nonspecific	virRvi3	virRvi1	virRvi5
Gala	100	37.5	75	87.5
Geneva	0	50	0	0
Golden Delicious	75	50	87.5	87.5
OR45t132	0	0	0	37.5

'Gala' (no *R*-gene) and 'Golden Delicious' *(Rvi1)* leaves presented signs and symptoms of scab by all conidia suspensions **(Figure 16).** On the other hand, 'Geneva' *(Rvi3)* and OR45t132 *(Rvi5)* leaves were infected only by conidia collected from the same cultivar. Therefore, the four conidia suspensions represent three different pathogenicity profiles: the conidia from 'Gala' and 'Golden Delicious' belong to race (1); the conidia from 'Geneva' belongs to race (1, 3); and the conidia from OR45t132 to race (1, 5).

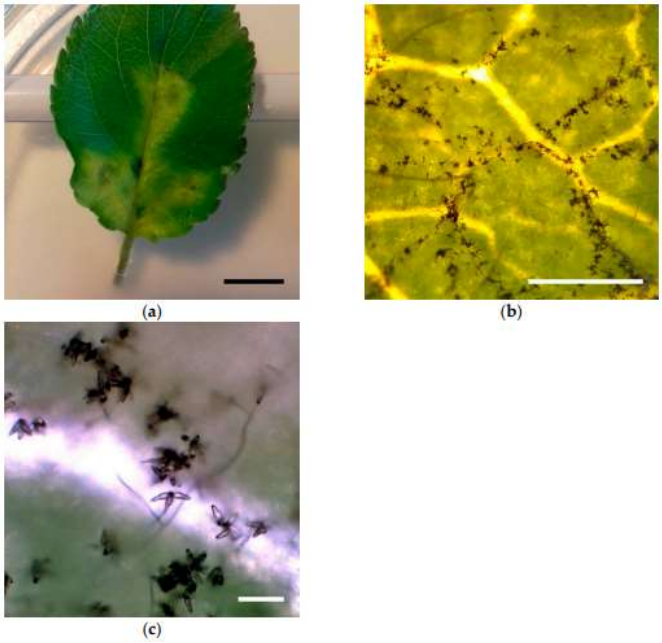

Figure 16. Symptoms caused by *V. inaequalis* on the detached leaves of 'Gala', 14 days post-inoculation: **A.** Sporulating lesions surrounded by chlorosis, scale = 5 mm; **B.** Conidia alongside running hyphae, forming a characteristic net-like or 'crisscross' shape, scale = mm, **C.** conidia developing on conidiophores in clusters, scale = um.

5.4.1. Germination rate

Same conidial suspensions were used to evaluate the germination rate of the spores in vitro **(Table 7).** 'Gala' showed the highest rate of germination (81%) while OR45t132 the lowest one (59%) which is in line with the results obtained in the curve of conidia production assessed by using cellophane.

Table 7. Germination rate of *V. inaequalis* spores from four scab race indicator cultivars.

Cultivar	Germinated spores (%)
'Gala'	81
'Geneva'	70
'Golden Delicious'	62
OR45t132	59

6. DISCUSSION

One of the main challenges of the twenty-first century is to provide high quality crops for its increasing population. In case of international market for fresh apple, it requires high aesthetic fruit characteristics in terms of size, appearance, and maturity (UNECE, 2020). Growers struggle to meet these market demands when orchards exhibit symptoms and signs of diseases, such as apple scab.

Nowadays, fungicide application is the main management method in order to control the spread of the apple scab pathogen and produce commercially acceptable fruits, with up to twenty sprayings every growing season (Zelmene et al., 2022). However, an intensive chemical control has become questionable due to the current regulations and the adverse effects reported, including negative impacts on the environment, workers and consumers health, and the emergence of populations resistant to systemic fungicides e.g., dodine and Qols (Fontaine et al., 2009; Mondino et al., 2015).

Therefore, a long-term strategy should be applied to provide high-quality fresh products to future generations taking into consideration economic viability and ecological sustainability (Kaur and Kaur, 2020). The first step toward this strategy consists in using existing scab-resistant cultivars and breeding new ones (Ward, 2012), for which it is important to understand the interaction between *V. inaequalis* and its host. However, the complex system *V. inaequalis – Malus* is dynamic, is conditionate to evolutionary pressures (Jones and Dangl, 2006), and has evolved through time (Bus et al., 2011). Thus, keeping on collecting new data about *V. inaequalis – Malus* makes it possible to produce more eco-friendly, robust, and resilient apple fruits on the long run (Höfer et al., 2021).

The current research aimed to quantify different fitness parameters of four *V. inaequalis* races of an apple cultivar collection (Budapest, Hungary) and to better understand the relationship between these parameters by using traditional methods. The race isolates are originated from four scab race indicator cultivars, 'Golden Delicious' *(Rvi1),* 'Geneva' (*Rvi3*, complex), OR45t132 *(Rvi5),* and 'Gala' (no *R*-gene) thus representing the corresponding pathogen races.

MacHardy et al. (2001) describes parasitic fitness as the ability of a parasitic genotype or population to persist successfully over time. Both types of fitness are based on reproductive success and/or survival of the pathogen. Furthermore, they emphasized the fact that *V. inaequalis* is a successful parasite on apple because it has accumulated numerous parasitic fitness attributes that enable it to infect and reproduce, but with little apparent damage to the host. Moreover, apple survives because it has accumulated numerous biological fitness attributes that enable it to cope with the parasite: scab is not life threatening, and a tree can withstand numerous infections and bear fruit (MacHardy et al., 2001).

Several research have been focused to measure *V. inaequalis* fitness by different traditional and new approaches, including: assessment of microscope (presence of conidia) and macroscope (symptoms range) responses on infected leaves in vivo (Clark et al., 2014), quantification of infection by real-time polymerase chain reaction (qPCR) (Gusberti et al., 2012), and tests of fungicide resistance *in vitro* (Cordero-Limon, 2018). Nowadays, traditional methods to evaluate apple scab pathogen growth (mycelium growth) and its capacity to reproduce (conidia production and its ability to germinate) are still widely used, but generally they require more time and labor (Aylor and Sanogo, 1997; Parker et al., 1995).

In this work we selected traditional methods, including morphological characterization of isolates, mycelium growth of *V. inaequalis* isolates under different lab conditions, cellophane method for conidia production, and pathogenicity testing by DLA. The cellophane experiment aimed to produce spores at the laboratory and evaluate its dynamics in 27 days. On the other hand, DLA was carried out to evaluate microscope (structures from the interaction between *V. inaequalis* – apple) and macroscope (symptoms range) responses on leaves infected with naturally collected spores, and quantify its germination rate.

Traditional methods are suitable nowadays because they are relatively easier, less expensive than immunological and molecular genetic methods, and do not need sophisticated equipment. On the other hand, immunological and molecular genetic approaches require sophisticated equipment, and take time if they are not standardized. In the case of characterization of *V. inaequalis* monosporic isolates, successful studies are based on the easiness to set and read morphological features, characterized by several morphological and cultural features of isolates (Nasonov, 2020).

Furthermore, Kirkham (1957) identified morphotypes of apple scab pathogen by evaluating fungal aerial mycelium, and their capacity of sporulation. His findings showed that there was a relationship between resistance of the host source, host range, stability of cultural characters, and pathogenicity of *V. inaequalis* isolates (Kirkham, 1957). This is in line with our experiment in which we found significative differences among groups of fungal monosporic isolates ('Gala', 'Golden Delicious', 'Geneva' cultivars, and OR45t132) in terms of color ($p<0.005$), density ($p<0.005$), and texture ($p<0.05$) of the main air mycelium.

This outcome provided an initial hint on the differences among groups of fungal isolates. There were some differences between our experiment and Nasonov's (2020) because we only determined the morphological characteristics of the air mycelium on sixty isolates. On the other hand, Nasonov (2020) took into consideration both features of air mycelium and central tubercle in a much larger sample of isolates ($n = 250$).

Furthermore, results obtained from the evaluation of mycelium growth between *V. inaequalis* isolates ('Gala', 'Golden Delicious', 'Geneva', and OR45t132) under different conditions (T1, T2, and T3) showed significant

difference in average growth (mm) between the investigated isolates from different host ($p<0.0001$). In every treatment, 'Gala' was larger than OR45t132 isolates ($p < 0.001$). This pattern was repetitive even when H_2O_2 was added as stressor (T3), showing great fitness capacity of 'Gala' isolates over the other groups under different temperature and stress conditions.

Adding a stressor in the culture medium had the aim to mimic natural conditions for *V. inaequalis.* This is due to some apple cultivars produce reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), and superoxide (O_2^-) in response to scab infection. These molecules can give rise to free oxygen radicals $HO_2^{\cdot}$ and $OH^{\cdot}$ which are even more reactive and play a role in the hypersensitive response in cultivars with certain major *R* genes (Lamb and Dixon, 2003; Torres et al., 2006).

Furthermore, in our experiments we made use of another opportunity to mimic *V. inaequalis* infection at the laboratory by growing the fungus on cellophane discs. This is possible due to in a normal progression of scab infection, *V. inaequalis* spores can germinate, penetrate, and colonize the leaf or fruit surface, as a result, the hyphae differentiate and form stromata between the cuticle and the epidermal outer wall. Then, the subcuticular stromata produce conidiophores and conidia which are released by breaking the leaf cuticle. Cellophane mimics the role of leaf cuticle, allowing scab infection follows its regular progression *in vitro* (Kucheryava et al., 2008). In order to achieve optimal conidia mass production from mycelium, Parker et al (1995) described that it is necessary to combine the technique of cellophane-cover agar surfaces with the triggering effect of UV light (Parker et al., 1995).

According to Julien and Spangelo (1957), cultures of different *V. inaequalis* isolates vary widely in pathogenicity, growth habits, and conidium production. Additionally, Ross (1974) observed that there was a correlation between sporulation among individual cultures and races of fungus, and also optimum conidium production coincided with the establishment of good mycelium growth. This strong positive correlation ($R = 0.89$) between mycelium growth and conidia spore production was seen in our experiment as well.

Following the course of experiments, outcomes of the conidia method showed that 'Gala' had the highest spore density yield followed by 'Geneva', and then OR45t132 ($p < 0.001$). These results are similar to the ones reported by Ross (1974) who described that under conditions favorable for sporulation, the level of conidium production *in vitro* of race 1 (e.g., 'Gala', Papp et al., 2022) and race 3 (e.g., 'Geneva', Buss, 2011) remained high in all experiments whereas most of the other cultures did not maintain a consistent level of sporulation. His experiment includes variables such as temperature and concentration of culture medium nutrients (glucose, nitrogen, KH2PO4, and MgSO4).

Additionally, it was seen under microscope that conidia spores presented typical shape, but in the case of 'Geneva' they seemed particularly elongated. There are no previous reports of this abnormal elongated shape of *V.*

inaequalis conidia spores. However, Golan et al. (2023) studied *Alternaria solani* spores and their findings showed that there is disadvantage of large conidia spores over the small ones. Large sized conidia landed sooner, moved over shorter distances, and survived shortly as compared to the smaller conidia. In addition, larger conidia were related to decline germinability when exposed to atmospheric conditions. They hypothesized that this data relating larger spore size to poorer survival in the atmosphere and shorter distances travelled can likely be translated to other fungal species (Golan et al., 2023). Therefore, taking into account this report, we conjectured that the elongated spore shape might mean poor fitness attributes for the 'Geneva' group in natural environment. Further analyses are needed to confirm it.

Unfortunately, 'Golden Delicious' was excluded from this analysis due to failure to sporulate regardless several attempts of standardization with different isolates. This is not an isolated report, an experiment carried out by Julien and Spangelo (1957) showed that *V. inaequalis* isolate from Summerland cultivar, originated from the cross McIntosh X Golden Delicious (Lapins, 1971), did not sporulate on any medium even though it presented good mycelium growth (Julien and Spangelo, 1957).

To overcome this inconvenience, DLA experiment was carried out in which four conidia suspensions were obtained by harvesting *V. inaequalis* conidia from naturally infected leaves of 'Gala', 'Geneva', 'Golden Delicious', and OR45t132, and then, detached leaves of the same four cultivars were drop inoculated with each suspension. All conidia suspensions caused obvious scab symptoms on their original host cultivars, fulfilling Koch's postulates. All conidia suspensions caused infections on 'Gala' and 'Golden Delicious' *(Rvi1),* which is in line with the GFG model where virulent pathogens are also capable of infecting non-resistant hosts (Laine and Tellier, 2008). Thus, all conidia suspensions can be designated to race (1). In contrast to this, no successful infection was detected on 'Geneva' *(Rvi3)* or OR45t132 *(Rvi5)* when the inoculum had a different host of origin. Only the conidia collected from the same cultivar caused symptoms on either 'Geneva' or OR45t132. Therefore, the four conidia suspensions represent three different pathogenicity profiles based on the capacity to infect the cultivar samples: the conidia from 'Gala' and 'Golden Delicious' belong to race (1); the conidia from 'Geneva' belongs to race (1, 3); and the conidia from OR45t132 to race (1, 5).

These pathogenicity profiles are in line with genetic data obtained by Papp (2022) from the same isolates used in the present work. Genetic data showed that isolates from 'Gala' and 'Golden Delicious' did not show significant genetic differences, and DLA indicated that their conidia suspensions were no specific to their respective hosts. On the other hand, OR45t132 was the genetically most distinct and least diverse group, and DLA showed that its conidia suspension was specific to its host. Regarding 'Geneva', this subpopulation was genetically less distinct from the rest of the population than OR, and DLA showed once again that its conidia suspension was specific to its host.

Furthermore, DLA experiment provided a hint of resistance breakdown in the studied cultivars. 'Golden Delicious' carrying the *Rvi1* gene have been grown for more than a century worldwide and was considered moderately resistant to scab in the beginning of the 20th century. Its resistance however completely diminished during its long history of cultivation. In some studies, it produced even more scab symptoms after artificial inoculation (with non-specific inoculum) than the susceptible control cultivar (Parisi et al., 2004).

According to Ignatov and Bodishevskaya (2011), the apple industry still relies on monogenetic resistance. The *Rvi6* (e.g., 'Priscilla') resistance gene is the basis for almost all contemporary scab-resistant apple cultivars. The *Rvi1* (e.g., 'Golden Delicious') gene is also present in the growing, although it is considered highly ineffective. However, its contribution to some cultivar's resistance, also carrying Rvi6, is still debated. Even in countries like Switzerland and Germany, where integrated production systems are well established, scab-resistant apples account for no more than 5–6% of the market; in Italy, scab-resistant apples account for less than 1%. On the global market, none of the released scab-resistant cultivars could compete with standard cultivars (Ignatov and Bodishevskaya, 2011). Relying on a single *R*-gene represents a risk on the long run. This high evolutionary pressure on a given *V. inaequalis* population can result in genetic mutations and/or the emergence of new races able to avoid recognition by the plant (Masny, 2017; Zelmene et al., 2022).

Genetically uniform host populations are more vulnerable to losing their resistance due to the evolutionary processes of fungal pathogens, and thus there have been many documented cases of *V. inaequalis* overcoming apple scab resistance in monocultures (Bus et al., 2011). Diversifying the cultivar use, hinders the establishment of a single highly virulent *Venturia* strain and is advantageous in the growing. Although the diversification of the pathogen race structure in a few years, and thus the decrease of population level resistance is common in mixed orchards/cultivar collections (Papp et al. 2020). For instance, in a study carried out in Germany by Höfer et al. (2021), researchers used a set of 680 apple cultivars from the Fruit Gene bank in Dresden Pillnitz to evaluate the incidence of scab in two consecutive years. Findings showed that the incidence of scab increased significantly in the second year. 60 cultivars with very low incidence in both years of scab were analyzed with molecular markers linked to known resistance genes. Most of them were identified to express alleles or combinations of alleles linked to *Rvi2, Rvi4, Rvi6, Rvi13, Rvi14,* or *Rvi17*. It has been hypothesized that the effect of host population diversity on scab resistance is primarily guided by major *R*-genes (Papp et al. 2022). The information on resistance/susceptibility of fruit genetic resources towards economically important diseases is important for breeding and for replanting traditional cultivars (Höfer et al., 2021).

We concluded that the most significant differences in terms of fitness attributes relied on 'Gala' and OR45t132. We found significative differences among groups of fungal monosporic isolates ('Gala', 'Golden Delicious', 'Geneva' cultivars, and OR45t132) in terms of color ($p<0.005$), density ($p<0.005$), and texture ($p<0.05$) of the main air

mycelium. The outcome of mycelium growth evaluation under different conditions (T1 – 19ºC, T2 – 14ºC, and T3 – H_2O_2) showed that 'Gala' was larger than OR45t132 isolates in every case ($p < 0.001$), even when the stressor (T3) was added. This was in line with the outcome of cellophane method which showed that 'Gala' isolates reached the highest spore density yields among the tested groups ($p<0.001$). Therefore, 'Gala' isolates showed high fitness attributes regarding vegetative growth and reproduction success. Following the course of experiments, DLA conidial suspensions were used to evaluate the germination rate of the spores *in vitro.* Once again, 'Gala' (race 1) had the highest rate of germination which is according with the curve of conidial production from isolates. On the other hand, OR45t132 isolates had the smallest mycelium growth and lowest spore density yield thus presenting weak fitness attributes regarding vegetative growth and reproduction success, respectively. Conidial suspension of OR45t132 (race 1,5) had the lowest germination rate among groups, which supports its poor reproduction success compared to 'Gala'.

The fitness attributes and pathogenicity of *V. inaequalis* isolates evaluated in this study are subject to constant change through time due to the dynamism of the *V. inaequalis – Malus* system given by environmental and genetic factors. Therefore, characterization of different races of *V. inaequalis*, differential host, and further studies of *V. inaequalis – Malus* system are need to be reviewed constantly to obtain updated information. In this way, the scientific community would be able to identify the races of *V. inaequalis* that could defeat an individual source of resistance, and then develop apple breeding programs to produce high quality fruits in a more ecofriendly way.

7. CONCLUSIONS

- Considering that the fitness ability of a parasitic individual consists of persisting successfully over time in terms of reproductive success and survival, we observed that the most significant differences in terms of fitness attributes relied on 'Gala' and OR45t132. 'Gala' had larger mycelium growth under different conditions of temperature and stress, and also had the highest spore density yield among the tested groups. On the other hand, OR45t132 isolates had smaller mycelium growth and the lowest spore density yield. Therefore, 'Gala' isolates showed high fitness attributes regarding vegetative growth and reproduction success.
- Based on the signs and symptoms of scab on the apple leaves during the DLA, the four conidia suspensions represent three different pathogenicity profiles: the conidia from 'Gala' and 'Golden Delicious' belong to race (1); the conidia from 'Geneva' belongs to race (1, 3); and the conidia from OR45t132 to race (1, 5). All conidia suspensions caused infections on 'Gala' and 'Golden Delicious' leaves during the DLA, but their conidial suspensions were unable to infect the resistant cultivars ('Geneva' and OR45t132). On the contrary, the OR45t132 isolate (race 1, 5) and the 'Geneva' isolate (race 1, 3) could infect the leaves of susceptible 'Gala' and 'Golden Delicious' cultivars although not the other resistant cultivar.
- The fitness attributes and pathogenicity of *V. inaequalis* isolates evaluated in this study are subject to constant change through time due to the dynamism of the *V. inaequalis – Malus* system given by environmental and genetic factors. Therefore, characterization of different races of *V. inaequalis*, differential host, and further studies of *V. inaequalis – Malus* system are required to be reviewed constantly to obtain updated information that allow breeders to produce high quality fruits in a more ecofriendly way.

8. SUMMARY

Apple scab, caused by the ascomycete fungus *V. inaequalis*, is an economically devastating disease of apples especially in production regions where moist conditions and cool temperatures prevail during the spring months. The most visible symptoms occur on leaves and fruits, when heavily infected they may drop prematurely. Currently, fungicide application is the main management method in order to control the spread of the apple scab pathogen and produce commercially acceptable fruits, with up to twenty sprayings every growing season. The intensive chemical control has become questionable due to the current regulations and the adverse effects reported, including negative impacts on the environment, workers and consumers health, and the emergence of populations resistant to systemic fungicides e.g., dodine and Qols. Therefore, a long-term strategy should be applied to provide high-quality fresh products to future generations taking into consideration economic viability and ecological sustainability. The first step toward this strategy consists in using existing scab-resistant cultivars and breeding new ones, for which it is important to understand the interaction between *V. inaequalis* and its host. However, the complex system *V. inaequalis – Malus* is dynamic, is conditionate to evolutionary pressures, and has evolved through time. Thus, keeping on collecting new data about *V. inaequalis – Malus* makes it possible to produce more eco-friendly, robust, and resilient apple fruits on the long run.

The current research aimed to quantify different fitness parameters of four *V. inaequalis* races of an apple cultivar collection (Budapest, Hungary) and to better understand the relationship between these parameters by using traditional methods. The race isolates are originated from four scab race indicator cultivars, 'Gala' (no *R*-gene), 'Golden Delicious' *(Rvi1),* 'Geneva' (*Rvi3*, complex), and OR45t132 *(Rvi5),* thus representing the corresponding pathogen races. We evaluated parameters that play a major role in the pathogen's survival ability through time (fitness), including vegetative growth and reproductive success, and also the ability to infect different apple cultivars *in vitro* (pathogenicity).

The steps carried out at the laboratory included the morphological characterization of isolates, mycelium growth of *V. inaequalis* isolates under different lab conditions, cellophane method for conidia production, and pathogenicity testing by detached leaf assay - DLA. The cellophane experiment aimed to produce asexual conidia spores *in vitro* and evaluate its dynamics in 27 days in order to evaluate the reproduction success of the race isolates. On the other hand, DLA was carried out to evaluate *V. inaequalis* pathogenicity by observing microscope (structures from the interaction between fungus– apple) and macroscope (symptoms range) responses on apple leaves infected *in vitro.*

We observed that the most significant differences in terms of fitness attributes relied on 'Gala' and OR45t132. We found significative differences among groups of fungal monosporic isolates ('Gala', 'Golden Delicious', 'Geneva' cultivars, and OR45t132) in terms of color ($p<0.005$), density ($p<0.005$), and texture ($p<0.05$) of the main air

mycelium. The outcome of mycelium growth evaluation under different conditions (T1 – 19ºC, T2 – 14ºC, and T3 – H_2O_2) showed that 'Gala' was larger than OR45t132 isolates in every case ($p < 0.001$), even when the stressor (T3) was added. This outcome showed a higher vegetative growth capacity of 'Gala' isolates over the other groups under different temperature and stress conditions. This was in line with the outcome of cellophane method which showed that 'Gala' isolates reached the highest spore density yield followed by 'Geneva', and then OR45t132 ($p < 0.001$). Therefore, 'Gala' isolates showed high fitness attributes regarding reproduction success. Following the course of experiments, DLA conidial suspensions were used to evaluate the germination rate of the spores *in vitro*. Once again, 'Gala' had the highest rate of germination which is according with the curve of conidial production from isolates. On the other hand, OR45t132 isolates had the smallest mycelium growth and lowest spore density yield thus presenting weak fitness attributes regarding vegetative growth and reproduction success, respectively. Conidial suspension of OR45t132 had the lowest germination rate among groups, which supports its poor reproduction capacity compared to 'Gala'.

Furthermore, based on the signs and symptoms of scab on the apple leaves during the DLA, the four conidia suspensions represent three different pathogenicity profiles: the conidia from 'Gala' and 'Golden Delicious' belong to race (1); the conidia from 'Geneva' belongs to race (1, 3); and the conidia from OR45t132 to race (1, 5). All conidia suspensions caused infections on 'Gala' and 'Golden Delicious' leaves during the DLA, but their conidial suspensions were unable to infect the resistant cultivars ('Geneva' and OR45t132). On the contrary, the OR45t132 isolate (race 1, 5) and the 'Geneva' isolate (race 1, 3) could infect the leaves of susceptible 'Gala' and 'Golden Delicious' cultivars although not the other resistant cultivar.

The fitness attributes and pathogenicity of *V. inaequalis* isolates evaluated in this study are subject to constant change through time due to the dynamism of the *V. inaequalis – Malus* system given by environmental and genetic factors. Therefore, characterization of different races of *V. inaequalis*, differential host, and further studies of *V. inaequalis – Malus* system are required to be reviewed constantly to obtain updated information. In this way, the scientific community would be able to identify the races of *V. inaequalis* that could defeat an individual source of resistance, and then develop apple breeding programs to produce high quality fruits in a more ecofriendly way.

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11. ATTACHMENTS

DECLARATION

on authenticity and public assess of mater's thesis

Student's name:	Cristina Cumanda Paredes Machado
Student's Neptun ID:	HPCUZR
Title of the document:	Fitness and pathogenicity of *Venturia inaequalis* isolates
Year of publication:	2023
Department:	Fruit Growing

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As a supervisor of Cristina Cumanda Paredes Machado (NEPTUN ID: HPCUZR), I here declare that the final master's thesis has been reviewed by me, the student was informed about the requirements of literary sources management and its legal and ethical rules.

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