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Phenotypic characterization of the interaction between *Plasmopara viticola* and *Vitis vinifera*.

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1.INTRODUCTION

1.1 Plasmopara viticola

Plasmopara viticola (Berk. and Curt.) Berl. and de Toni is an obligate biotrophic oomycete which causes downy mildew, the most destructive fungal disease of grapevine. *P. viticola* can severely infect all the green parts of the plant and its particularly damaging on leaves, inflorescences and bunches.

The seriousness of damage caused by *P. viticola* is influenced by weather conditions, which favour the infections in presence of high humidity and low temperatures in late spring-summer, leading to numerous infection cycles. *P. viticola* is, in fact, a polycyclic pathogen.

P. viticola originated in North America, where it spread from wild grapes to cultivated vineyards even before 1834. The pathogen spread out in USA during 1860 and in seven years the disease caused serious loss in wet heat country of USA. The disease was not reported in Europe until 1878, when it was apparently introduced in France on a grape cultivar imported from the USA for use as a rootstock resistant to grape phylloxera. During 1879, disease spread out in different French areal and in autumn it was discovered by Professor Pirotta in S. Giuletta, nearby Voghera, located in Lombardy (Ferraris, 1913). After few months, Saccardo noticed *P. viticola* in Veneto (Galet, 1977). During 1880, disease spread out in northern Italy and in Austria; in 1881-1882 it was reported in Switzerland, Germany, Spain, Turkey, Russia, Africa and Asia Minor.

To the rapid spread, was coupled the capability to cause serious losses, due to cluster destruction and loss of vine foliage.

During the first years of its introduction in Europe, the disease did not attract the attention of viticulturists, due to its appearance limited to autumn, that caused only early fall leaves (Ferraris, 1913). The sudden appearance of the disease in spring leading to the infection to bunches, especially in form of brown rot, lead to serious yield drops (Ferraris, 1926). In fact when meteorological conditions favor the infections of flowers or young berries, crop losses from 50% to 100% occur in absence of an adequate control of the disease. .2

1.2 TAXONOMY

P. viticola was first described in 1834 by Schweintz and taxonomically classified as *Botrytis cana* LK., a synonym of *Botrytis cinerea.* Berkeley and Curtis later described the organism as *B. viticola* (1855). De Bary transferred the pathogen to a new genus and described it as *Peronospora viticola* (1863). Berlese and de Toni in 1888 redescribed the pathogen as *Plasmopara viticola* (Berk. *et* Curt.) Berlese and De Toni (Saccardo, 1888).

P. viticola has long been included in the fungi Kingdom *Mycota*, in the division *Eumycota*, class *Oomycetes* which includes fungi provided with heterokont flagellation and formation of oospores through sexual reproduction (Webster J., 1980). The *Oomycetes* possess peculiar characteristics, that spearate them from the true fungi such as: the sexual structures, the oospores; the presence of cellulose in the cell wall, instead of chitin; and the vegetative stage, consisting of coenocytic hyphae (hyphae without septa) which contain diploid nuclei. These peculiarity made it necessary in-depth investigations on this taxonomic group which

led Dick (2001) to propose the separation of *Oomycetes* from the *Eumycota* and the collocation into the class *Peronosporomycetes* of the new Kingdom of *Straminipila.* This Kingdom encompasses biflagellate fungi, diatoms, chrysophytes, xanthophytes, phaeophytes etc. (Dick, 2001). The organisms belonging to the *Straminipila* possess zoospores with an anteriourly directed flagellum named 'straminipilous', for the tubular tripartite hairs (TTHs) which pulls the zoospores through the water. The ribosomal RNA sequencing established that these different organisms have a monophyletic origin for the ontogeny and morphology of the TTHs which are too elaborate and too costant to support a hypothesis of convergent evolution. Obtained these results, Dick affirmed that these organisms are encompassed in a unique Kingdom, and therefore having developed from a common heterotrophic ancestor, refuting the hypothesis proposed by Cavalier Smith (1986) according to which they originated from an endosymbiont photosynthetic ancestor.

The *Straminipila* Kingdom includes organisms characterized by mitochondria with tubular christae (on the contrary the majority of higher plants and animal are characterized by lamellar mitochondrial cristae), by lysine synthesis pathway (diaminopimelic acid pathway) and, if photosyinthetic, by plastids including chlorophyll c and not chlorophyll b as in green plants.

According to Dick (2001), in the *Straminipila* Kingdom, *P. viticola* belongs to the *Peronosporomycetes* class, located in the *sub-phylum* Straminipilous fungi, *Peronosporomycotina* (Table 1.1).

The diploid phase in these fungi is typical of vegetative stage, in contrast with *Eumycota.*

The mitosis exclusively occurs during the diploid phase of the cycle and it represents the distinctive characteristic of this class, in fact in the other classes of *Straminipila* (*Labyrinthista, Hypochytriomycetes)* the mitosis occurs during aploid phase.

Other typical characteristics of *Peronosporomycetes* are: cruciform meiosis in a nuclear persistent membrane; multiple and simultaneous meiosis, in coenocytic gametangia; the absence of flagellate gametes; formation of oospores in oogonia.

Table 1.1- Classification of *Peronosporomycetes* according to Dick (2001).

SUBCLASS	**ORDERS**	**FAMILY**	**MAIN GENUS**
Peronosporomycetidae	*Peronosporales*	*Peronosporaceae*	*Peronospora*
			Plasmopara
			Bremia
		Albuginaceae	*Albugo*
	Pythiales	*Pythiaceae*	*Pythium*
			Phytophtora
		Pythiogetonaceae	
Saprolegniomycetidae	*Saprolegniales*	*Saprolegniaceae*	*Saprolegnia*
		Leptolegniaceae	
	Leptomiales	*Leptomiaceae*	
		Apodachlyellaceae	
		Leptolegniellaceae	

	Sclerosporales	*Sclerosporaceae*	*Sclerospora*
		Verrucalvaceae	
Rhipidiomycetidae	*Rhipidiales*	*Rhipadiaceae*	

The class *Peronosporomycetes* is divided into three subclasses (*Peronosporomycetidae, Saprolegniomycetidae* and *Rhipidiomycetidae*): this division is fully supported by 18S rRNA, 28S rRNA and mitochondrial cytochrome c oxidase sequences respectively. The separation of the *Rhipidiomycetidae* from the *Peronosporomycetidae* remains uncertain. It has to be pointed out that since the advent of molecular phylogeny, taxonomy is in constant evolution, and indeed the placement of some genera such as *Phythophthora* and *Pythium* is under reconsideration (Thines *et al*., 2009).

The evolution of *Peronosporomycetes* started in the Tertiary era, when the higher plants started to produce secondary metabolites, able to protect the plant from fungal pathogen and parasites. These metabolites (flavonoids, alkaloids, sterols and essential oils) have no effect on straminipilous fungi.

The straminipilous fungi included into genera *Phytophthora, Plasmopara, Peronospora* and *Sclerospora*, have caused significant damages to crops over time, leading to the establishment of plant pathology and the development of chemical industry in agriculture.

However, the taxonomy is constantly evolving. Rouxel *et al.,* (2013) evidenced that downy mildew is not caused by a single species but rather by a complex of cryptic species, proved by genetic and morphological analysis. *P. viticola* is characterized by several host specific cryptic

species that could be considered as formae speciales: *P. viticola* f.sp. *riparia*, *P. viticola* f.sp. *aestivalis*, *P. viticola* f.sp. *vinifera*, *P. viticola* f.sp. *quinquefolia* (Rouxel *et al.,* 2013).

1.3 LIFE CYCLE

P. viticola is a polycyclic pathogen (Fig. 1.1) and an obligate parasite, able to survive in absence of the host by differentiating resting structures, the oospores.

The oospores were identified in 1880 by Millardet, whereas oogonia and antheridia were described a decade later by Pichi (1890). During late autumn-winter, the pathogen overwinters as oospores, differentiated by sexual reproduction. During spring, mature oospores germinate forming macrosporangia, which release biflagellate zoospores, devoid of wall. After a mobility period in the water layer present on the leaf surface, the zoospores, attracted to stomata, encyst and cause primary infections. During encystment, the two flagella detach, the cell wall is synthesized and a hypha penetrates through the stomatal aperture. The pathogen actively develops in the host tissues by forming an intercellular mcelium with the formation of haustoria, specialized feeding structures (Lamour and Kamoun, 2009).

At the end of the incubation period, the characteristic symptoms of the infection are visible and, in presence of high humidity, sporangiophores and sporangia are produced through stomatal openings.

The dissemination by wind or water-splash of these sporangia cause the secondary infections, with liberation of new zoospores, while in the leaf

parenchyma and in the other infected organs the pathogen differentiates the organs of sexual reproduction. The number of secondary infections depends on weather conditions (Vercesi *et al*., 2010).

Figure 1.1: *P. viticola* infection cycle (Belli, 2006)

1.3.1 Overwintering

Since the end of July, in the mesophyll of infected leaves, mycelium generates the male and female gametangia: oogonium and antheridium.

P. viticola is a heterotallic fungus: mating requires different sexually compatible types, called P1 and P2 (Scherer and Gisi, 2006).

The oogonia, initially devoid of septa, are considered female because they provide most of the cytoplasm to the oospores, which develop within oogonia.

Antheridia are separated from the mycelium by the apposition of a septum. The mature antheridium approaches to the oogonium following hormonal attraction and secretes adhesive material. In each gametangia meiosis occurs and the antheridium forms a fertilization tube (Dick, 2001). A single antheridial nucleus reaches the oosphere fusing with the oogonial nucleus, whereas the other nuclei degenerate (Burruano, 2000). During maturation, a multi-layered wall is established in the oospores.

1.3.2 Primary infections

The oospores are formed into the host tissues and overwinter on the surface litter.

Generally, the oospores germinate in spring, when there are frequent rains and mild temperatures. Each oospore produces a single macrosporangium (primary zoosporangia) from which biflagellate zoospores are differentiated through mitosis.

A septum divides the mature differentiated sporangium from the germination tube, while the oospore appeares completely empty and recognizable only by the oogonium and outer oospore walls (Vercesi *et al.,* 1999).

In the macrosporangium, 8 to 20 biflagellate zoospores are differentiated and released, in presence of a water film, through an apical operculum (Lafon and Bulit, 1981).

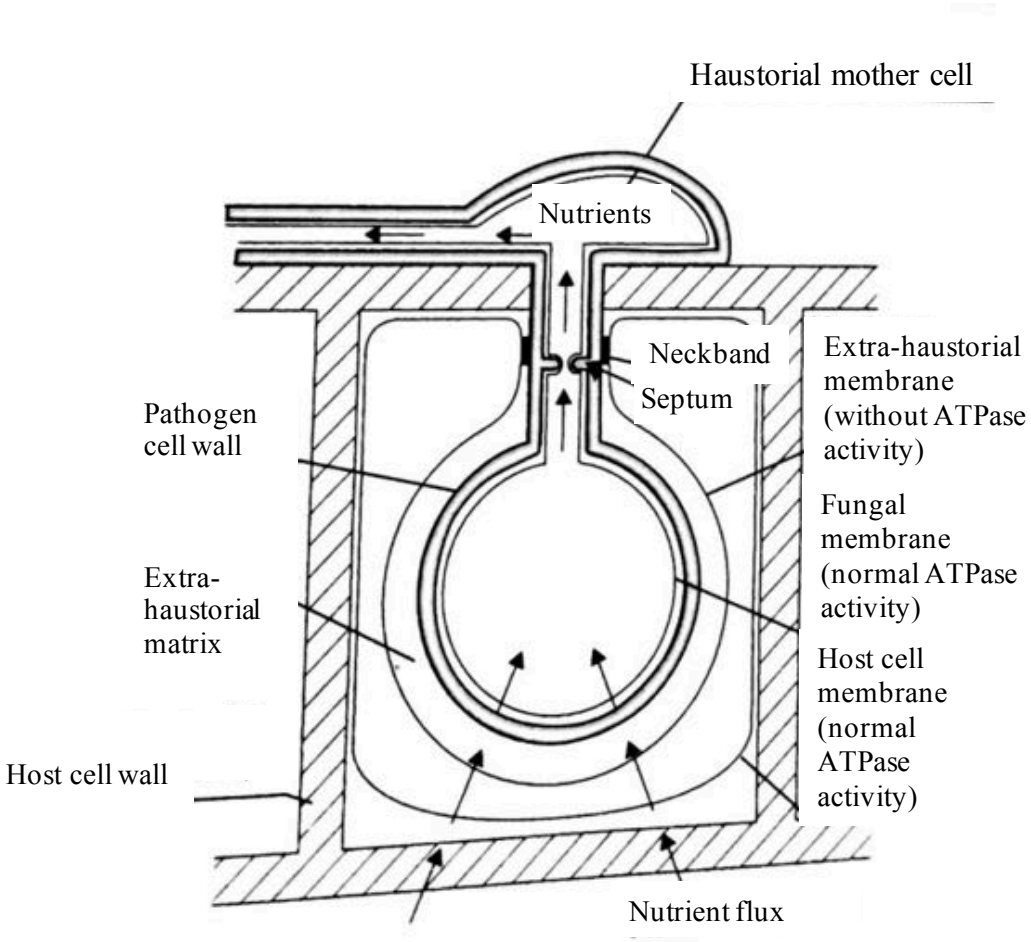
The zoospores are uninucleate cells, reniform and with a ventral groove. Two heterokont flagella emerge along this groove: the posterior is whiplash and the anterior is tinsel-type with tripartite hairs. The anteriorly directed flagellum pulls the zoospore through the water but its hydrodynamic thrust is reversed because of two rows of stiff tubular tripartite hairs (Dick, 2001). When the zoospores reach the host, they approach to stomata moving in water film on leaves following chemotactic urges, and encyst assuming a spherical form, detaching two flagella and rapidly forming the cell wall, and produce a germ tube to penetrate the host tissue trough stomata. The primary infections occur when the minimum atmospheric temperature is about 10°C, at least 10 mm of rain and that the vines have developed shoots measuring 10 cm (Baldacci, 1947; Baldacci and Refatti, 1956).

After penetration, *P. viticola* develops the mycelium in intercellular cavities of lacunos and palisade tissues and differentiates special interface within the living plant cells. These structures, called haustoria, are the sites of nutrients uptake from the host. The haustoria penetrate the cell walls, but not the protoplasts of the host cells.

The haustoria of *P. viticola* have a globular form and are enclosed by a wall characterized by two layers: one transparent and the other opaque to the electrons. Inside there are plasmalemma and, in the cytoplasm, mitochondria, vacuoles, lipids, endoplasmatic reticulum and many ribosomes (Amici *et al.,* 1968).

Tha haustorium invaginates the host plasmamembrane and it remains outside the physiological barrier of the host cell, to preserve the vitality of host cell, as the biotrophic pathogens need living tissue for growth and reproduction (Fig 1.2).

Figure 1.2: Haustorial structure in a host cell (Deacon, 2000)



The zone of separation between the host plasmamembrane and the pathogen consists of the fungal cell wall and the extra haustorial matrix (Hahn *et al.,* 1997a), which includes an amorphous mixture of components, such as carbohydrates and proteins, partly of pathogen but primarily of plant origin (Harder and Chong, 1991).

Haustoria are connected with the hypha through a slender neck that penetrates into the host cell forming a constriction with a 0,2 to 0,5 μm diameter, to cause a minimal damage to the cell. In this point the neck is encircled by callose deposition, synthesized by the plant.

The substrate translocation is controlled by the pathogen, thought active transport across cytoplasmic membrane generated by proton gradient (with ATPase) and the pathogen uptakes nutrient and canalises them into mycelium to maintain a gradient concentration between matrix and haustoria.

The nutrients are traslocated to the pathogen because of the absence of ATPase activity on extra haustorial membrane, which results permeable to substrates.

The pathogen infection alters the plant primary metabolism. A series of rapid changes results in a decline in photosynthesis and an increase in respiration, photorespiration and invertase enzyme activity. The host respiration does not involve Krebs cycle and glycolysis metabolic pathway but the pentose phosphate pathway, which results in the production of phenolic compounds, involved in the defence mechanism of the plants (Toffolatti, 2007).

The withdrawal of nutrients caused by the pathogen increases the demand

for assimilates. Pathogen infection often leads to the development of chlorotic and necrotic areas, close to the so-called "green islands", *i.e.* leaf parts characterized by a high level of chlorophyll and by more abundant chloroplasts. In these areas an increased photosynthesis occurs to compensate for decrease of photosynthetic tissue.

1.4 SYMPTOMS

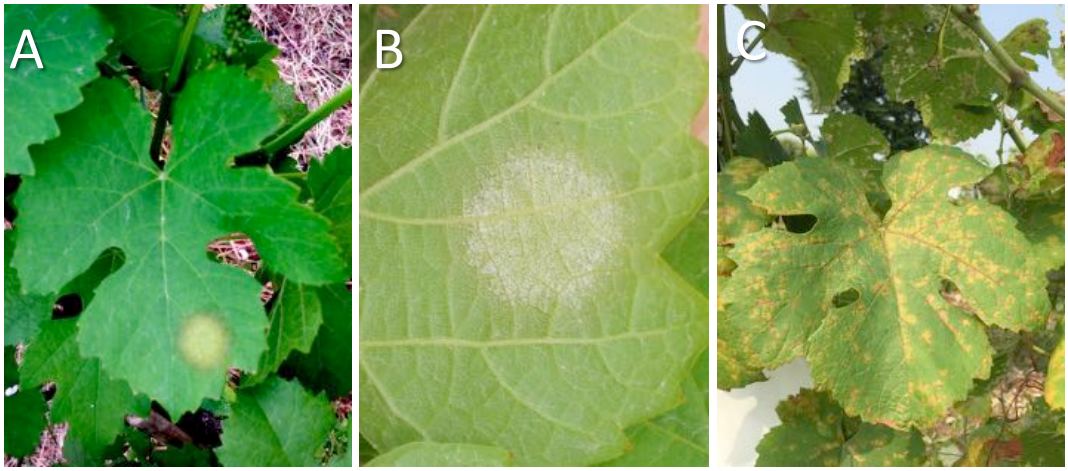
Leaves, tendrils, shoots, inflorescences, bunches can all be affected by *P. viticola*. The leaf infection causes qualitative yield drops due to the loss of photosynthetic activity leading to a lower sugar content in the bunches.

1.4.1 Symptoms on leaves

Leaves are more susceptible to infections during active growth.

The first symptoms of the disease, in spring, appear on the upper surface as circular oil spot, with yellow translucent aspect (Fig. 1.3A). In favourable weather conditions, white downy sporangiophores and sporangia develop from stomata on the underside of oil spots (Fig. 1.3B). Later, oil spots turn brown or reddish brown, dry out, and die. In late summer and in autumn, the symptoms appear as a mosaic of small, angular, yellow to reddish-brown lesions limited by veinlets.

Figure 1.3: Oil spot on the upper surface of leaf (A), fungal sporulation in the form of white mildew on the underside leaf (B) and mosaic symptoms (C).



1.4.2 Symptoms on shoots and tendrils

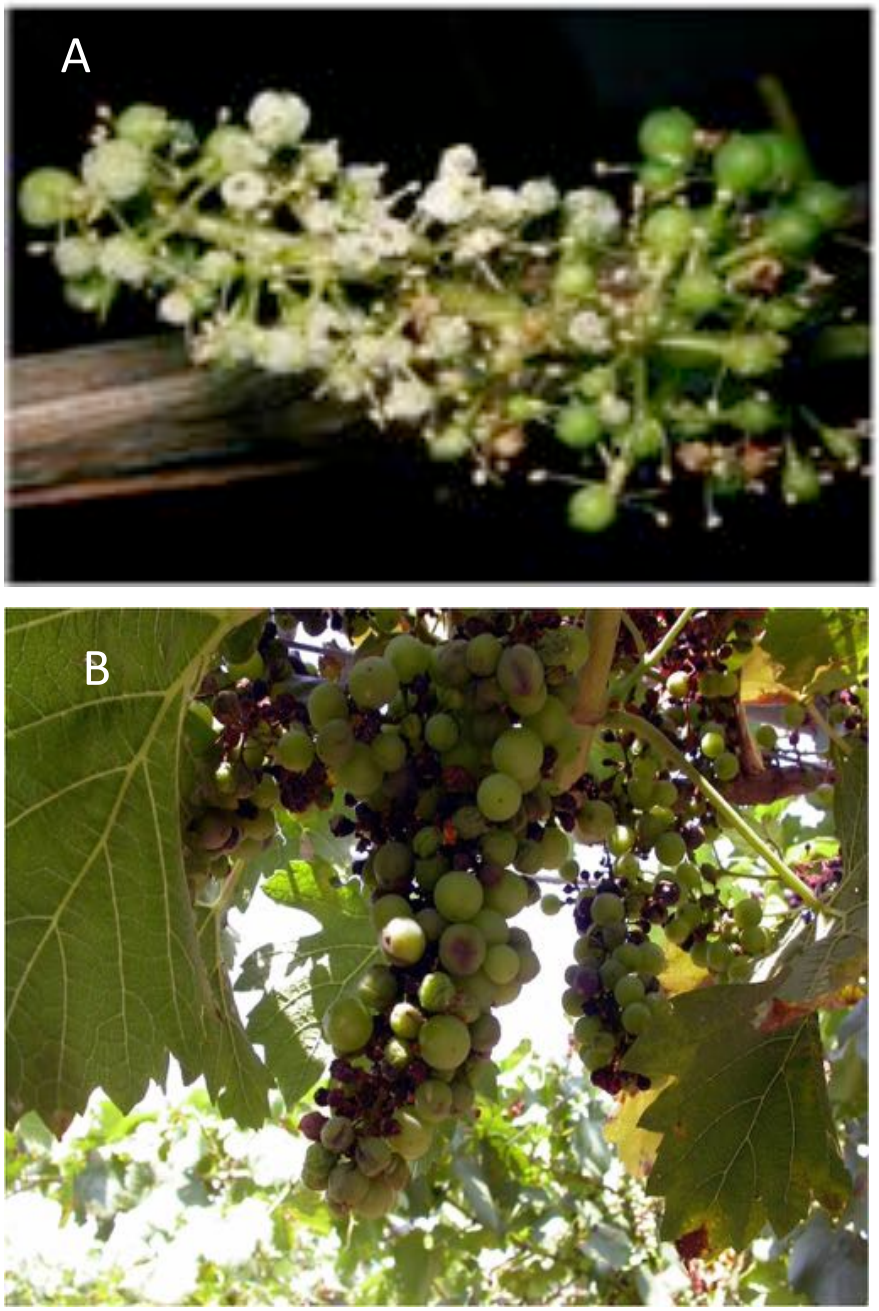
When young shoots and tendrils are infected, they turn brown and become stunted, distorted, and necrotic. Shoots and tendrils can be covered by downy mildew (Fig. 1.4). On shoots in phase of lignification the infection is less evident and appear as lesions of cortical tissues.

Figure 1.4: Symptoms on shoots

1.4.3 Symptoms on bunches

The infected bunches show two different symptoms in relation to the phenological phase. Young clusters are deformed and quickly covered by sporulation (Fig. 1.5A), in presence of high humidity, or dry up. Later on, when the stomata are closed, the clusters develop a purple coloration and dehydrate (brown rot) (Fig. 1.5B); the sporulation do not occurs on mature clusters because the stomata are no longer functional (Ferraris, 1926).

Figure 1.5: Symptoms on bunches: sporulation (A) and brown rot (B)



1.5 CONVENTIONAL DISEASE MANAGEMENT

The pathogen infections are usually prevented by using suitable cultural management practices and fungicide treatments.

The objectives of cultural management are to reduce the amount of inoculum and create conditions of lower receptivity of the plants (Lafon and Bulit, 1981).

In vineyards, microclimate depends on the vineyard layout and management and it can be controlled by choosing adequate training and pruning systems and regulating the vigor and nitrogen fertilization of the plants.

An efficient soil drainage and conservation tillage have to be promoted to prevent waterlogging, that favours the maturation and germination of oospores. For eliminating the resting structures, removal and burial of infected leaves is also suggested.

These strategies can contribute to slow down the disease level in vineyard, but they are not enough efficient in to keep the disease risk to an acceptable level and they have made it necessary the use of chemical products for the disease control.

Since the end of XIX century, when first chemical compounds were used for agricultural treatments, the phytosanitary practices have really changed, in particular as concerns the provided active substances.

Copper, discovered by Millardet in 1882, was the first fungicide active substance against *P. viticola*, used in vineyard to control downy mildew.

It was noticed by Millardet that the grapevines treated with a mixture of copper sulphate showed the absence of downy mildew symptoms (Millardet, 1885). This treatment, called Bordeaux mixture, obtained widespread employment and it is still used.

The active substances utilized at present in viticulture are, generally, really different from those that were early discovered and can be divided based on their translocation pattern after application.

Many fungicides remain on the surface of the plant tissues forming a protective barrier against the pathogen, acting preventively. The negative aspects of these fungicides are that they can be washed off by rain and do not protect vegetation formed after treatment.

These category of fungicides include copper (Cu^{2+}), which represent the most traditional and used active principle against downy mildew and the only active substance usable in organic agriculture (Perazzolli *et al.,* 2011). This substance interferes with numerous metabolic processes of the pathogen, with low risks to induce resistant strains. The huge advantages of the use the copper, namely high fungicidal activity and the low price, ensure that it is even extensively used in viticulture. This element may phytotoxic if used in presence of low temperatures and high relative humidity and at phenological stages, such as flowering and fruit setting.

The application of cupric compounds is recommended in the second part of the growing season of grapevine (Vercesi, 1999). In recent years, to solve the problem, specific formulations characterized by low phytotoxicity, such as copper oxychloride, have been created to allow copper usage also during the critical phenological stages of the grapevine (Gessler *et al*., 2011).

The evolution of the chemical control occurred with the introduction of synthetic organic fungicides, with a preventive activity. The first organic fungicides active against downy mildew commercially available were the dithiocarbamates which, unlike cupric compounds, present the great advantage of not being phytotoxic. However they induce in the host an excessive vegetative vigor, leading to an increased susceptibility to *Botrytis cinerea* Pers. infections. Moreover, they are toxic for the natural competitors of mites and favours their infestations (Posenato, 1994; Vettorello and Girolami, 1992) and can be harmful for human health. For that reason, their use is limited to the central part of the season. The dithiocarbamate used in Italy, alone or with other penetrant fungicides, are mancozeb and metiram. Another multi-site inhibitor used is the quinone dithianon.

Due to toxicologial issues, the use of phtalimides, introduced on the market after the dithiocarbamates, is currently prohibited, except for folpet which contains *P. viticola* and showed a successful activity towards *B. cinerea* and *Phomopsis viticola* (Sacc.), the excoriose agent.

The second significant revolution in the chemical control of downy mildew occurred introducing systemic and cytotropic active ingredients. They are characterized by a different mechanism of action, penetration and movement into the plant, but they share the ability to penetrate into the host tissues and exert their fungicidal activity when the infection is already in progress, resisting to atmospheric agents (Vercesi, 1999).

These category of products is preferably used during the highest infection risk period. But having a single or oligosite mechanism of action, they lead to high prababilty to select resistant pathogen strains. To avoid the

problem, these substances are used for a limited number of treatment, and in mixture with surface-acting fungicides, characterized by a multisite mode of action, or with single-site active substances characterized by a different mode of action, possibly in alternation.

The only systemic fungicide with a mode of action which does not imply the risk to select resistant strains is the phosphonate fosety-Al. This substance is able to move in acropetal and basipetal sense and can be used alone; it both acts directly against the pathogen and stimulates the plant defences (Belli, 2006). The other fungicides registered in Italy against the grapevine downy mildew agent are phenilamides (metalaxyl-M and benalaxyl-M), QoIs (pyraclostrobin, famoxadone, fenamidone), QiI (cyazofamid and amisulbrom), CAAs (dimethomorph, mandipropamid, iprovalicarb, benthiavalicarb, valiphenal), cymoxanil, fluopicolide, ametoctradin and zoxamide. The application of anti-resistance strategies is recommended for all the classes.

The phenilamides inhibit ribosomal RNA synthesis, specifically RNA polymerization (Gisi and Sierotzki, 2008).

The QoI (Quinone outside inhibitor) class encompasses the strobilurins, derived by a metabolite of basidiomycete *Strobilurus tenacellus* (Pers. ex Fr.) Singer. They were introduced into the marker at the middle of 1990's, but their use against *P. viticola* is unfortunately in decline due to the early appearance of resistance (Toffolatti and Vercesi, 2012). The they inhibit the electron transport at cytochrome b (complex III) by binding to the Qo site, the ubiquinol oxidizing pocket, which is located at the positive, outerside of the mitochondrial membrane (Gisi and Sierotzki, 2008). QiI fungicides (Quinone inside inhibitors), on the contrary, bind to the Qi

center of complex III, the site of ubiquinone reduction, and do not show cross resistance with QoIs (Mitani *et al*., 2001).

The CAA (carboxylic acid amides) group was officially established by FRAC (Fungicide Resistance Action Committee, www.frac.info) in 2005. The broad spectrum activity of CAA is specific for oomycetes, such as *P. viticola* and *Phytophthora* spp.

They act on sporangia and zoospore germination (Knauf-Beiter and Hermann, 2005) but not on the release and on the motility of them; the CAA in fact affect on the germ tube and mycelium growth (Cohen and Gisi, 2007; Toffolatti *et al.,* 2011). Therefore these fungicides show a high preventive action and curative activity including newly formed tissues in the particular case of iprovalicarb, characterized by a systemic and antisporulant activity. The latter is observed also in dimetomorph, benthiavalicarb and mandipropamid (Gisi *et al.,* 2007).

Cymoxanil exerts curative and protectant activity against *P. viticola*, by hampering the development of the vegetative structures and preventing hyphal development and zoospore release from sporangia. Its mode of action is still speculative and resistance is not stable (Toffolatti *et al*., 2014).

Fluopicolide belongs to a recently established chemical class, the acyl-picolides, able to affect the zoospore motility and hyphal growth through the delocalisation of spectrin-like proteins (Latorse *et al.*, 2006).It shows both preventive and curative activity.

Ametoctradin is a new fungicidal active ingredient. The innovative compound belongs to a new chemical class, the triazolopyrimidylamines.

Ametoctradin is a mitochondrial respiration inhibitor interfering with the complex III (complex bc1) in the electron transport chain of the pathogen, thus ATP synthesis in the fungal cells is inhibited. It is highly effective in inhibition of zoospore formation and release, zoosporangia release, motility, and germination. Ametoctradin is a non-systemic fungicide that remains primarily on the leaf surface where it is adsorbed (Merk *et al.,* 2011).

Zoxamide is highly effective against oomycetes, and used for foliar application. The fungicide causes mitotic arrest by binding to β-tubulin, inhibiting tubulin polymerization and cell division of the pathogen. Via this mechanism, zoxamide does not affect initial spore germination but inhibits germ-tube elongation of the pathogen, which is required for penetration through host tissue (Bi *et al,*. 2011).

1.6 INNOVATIVE DISEASES PRACTICES

The use of fungicides is necessary to prevent severe disease epidemics. However the laws are very restrictive for their registration and application to protect human health and the environment. Moreover the increased and prolonged use of single-site fungicides has selected resistant *P. viticola* strains. For these reasons, finding alternative strategies to reduce infections is almost a necessity.

1.6.1 Induction of resistance

Resistance inducers are organic and inorganic substances able to stimulate the plant defence system by catalysing resistance reactions in plants,

comparable to those caused by a pathogen infection (Kessmann *et al.,* 1994).

Induced resistance can be local or systemic: in the case of the local resistance, the response is induced at the site where the treatment is applied; in systemic resistance, the induced response and the point of induction do not correspond. The systemic acquired resistance (SAR), is a state of heightened defense that is activated throughout the plant following primary infection by pathogens that elicit tissue damage at the site of infection (Kunkel and Brooks, 2002).

SAR can be induced by the exposure of foliar tissues to abiotic or biotic elicitors and is dependent of the phytohormone salicylate (salicylic acid), and associated with the accumulation of pathogenesis-related (PR) proteins (Vallad and Goodman, 2004). Salicylic acid (SA) was found to accumulate at high levels in phloem exudate and SA level are correlated with expression of the SAR gene; SA accumulation is essential for SAR and is a transmissible signal (Neuenschwander *et al.*, 1995). Jasmonic acid (JA) and ethylene are alternative signals in the induction of resistance against microbial pathogens (Dong, 1998).

There are very few detected substances which could to have effect towards *P. viticola*. Benzothiadiazole (BTH) whose effect is the same that salicylic acid, induces genes of resistance activating defence reactions. Other resistance inducers are 3-DL-β-aminobutyric (BABA) (Cohen *et al.,* 1999), which has an effect on the activation of the jasmonic acid that induces callose deposition around the infection site (Hamiduzzaman *et al.,* 2005), chitosan, laminarin, acibenzolar-S-methyl (BTH), fosetyl-Al, plant extracts. These resistance inducers promote stomatal closure, expression

of defence genes, increased enzymatic activity and the accumulation of phytoalexins.

This defence strategy has some disadvantages in relation to *P. viticola* control: for example the induction of resistance requires very long time and the plants until that moment are unprotected. On the contrary, constitutively expressed defence traits are quickly activated (Heil and Baldwin, 2002).

Moreover this process is very costly for the plant at the level of its fitness: large quantity of resources are in fact allocated to induce resistance traits and are, therefore, unavailable for fitness processes such as growth and reproduction. Some compounds synthesized during resistance response are moreover toxic for the plant and they might request a further significant metabolic cost. Furthermore a specific defence reaction towards a specific pathogen might have no effect on a different pathogen (Heil and Baldwin, 2002). At present, no natural substances or plant strengthener has been proved to be effective against *P. viticola* under field conditions: the substances were satisfactory in laboratory or in greenhouse trials, but no effects could be observed under field conditions (Harm *et al.,* 2011).

1.6.2 Genetic resistance

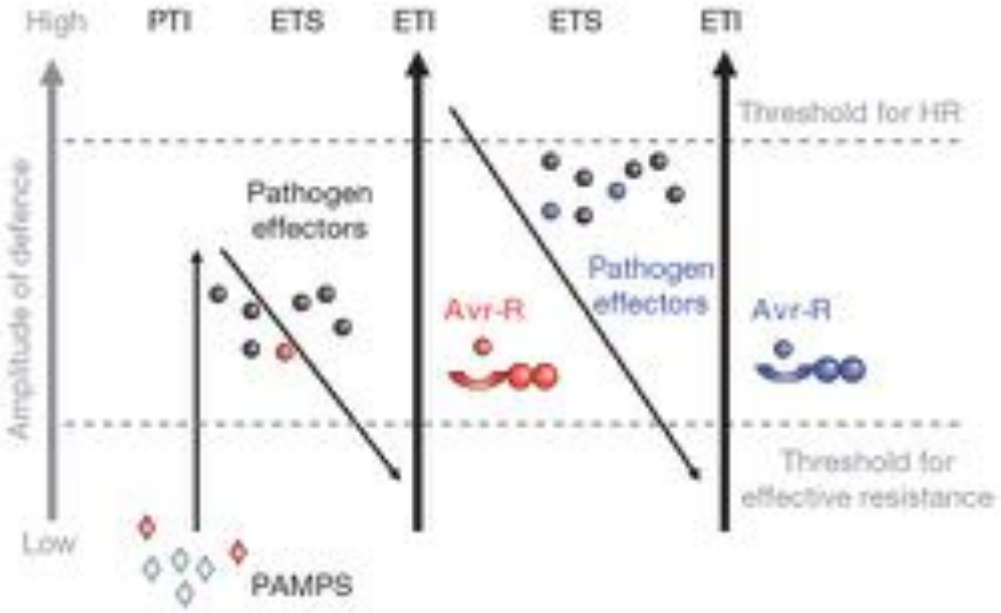
Plants are constantly in contact with pathogens, which penetrate into the hosts by leaf or root actively or through natural apertures such as stomata, as in the case of *P. viticola.*

Plants lack mobile defender cells and a somatic adaptive immune system. Instead, they rely on the innate immunity of each cell and on systemic signals emanating from infection sites (Jones and Dangl, 2006).

The immune system of an organism has been tailored through evolution by a long history of warfare with its invaders. Immune systems discriminate self from non-self, and activate tightly regulated pre- and post- invasion defense responses to minimize the damage inflicted by harmful agents (Coll *et al.,* 2011).

Oomycetes undergo a series of developmental stages throughout a successful infection cycle, including the formation of sporangia, release of motile zoospores, their encystment and germination to form hyphae, haustoria and, finally, sporangiophores (Birch and Cooke, 2004). The development of the pathogens into the hosts leads them to be constantly in contact with the host plasma membrane where there are receptors that recognize pathogen-associated molecular patterns (PAMPs) and microbe-associated molecular patterns (MAMPs), molecules which are essential for microbes to establish the infection. *P. viticola* PAMPs include β-glucan, a component of cell wall and the recognition occurs through invading haustoria (Birch *et al.,* 2006). The current view of the plant immune system can be represented as a four phased "zigzag" model proposed by Jones and Dangl (2006) (Fig. 1.6).

Figure 1.6: The "zigzag" model proposed by Jones and Dangl (2006).



PTI: Pamp-triggered immunity;

ETS: Effector-triggered susceptibility

ETI: Effector-triggered immunity

Phase 1.

The plant innate immunity is controlled by membrane-anchored pattern recognition receptor (PRRs) which recognize and bind to MAMPs or PAMPs resulting in PAMP- triggered immunity. The PRRs are leucine-rich repeat receptor kinase (LRR-RK) located in the plasma membrane.

The PAMP-triggered immunity (PTI) results from this recognition and consists in a cascade of reactions which induce the hypersensitive response (HR) and programmed cell death (PCD), a form of defence system mediated by mitogen-activated protein kinase (MAPK) cascades (MAPKKK, MAPKK, MAPK), and results in transcriptional activation of defence genes by plant-specific transcriptional regulators including

WRKY (Panstruga, 2009). This consists in the activation of a calcium burst: influx of Ca^{2+} in the cytosol, regulated by BIK1 proteins (Li *et al.,* 2014b). The calcium influx induces the alteration of the others membrane channel, causing the influx of H^+, efflux of K^+, Cl^- and NO_3^-, causing an alkalinisation.

Another response is the production of extracellular reactive oxygen species (ROS) by NDPH oxidase localized in the plasma membrane, named respiratory burst oxidase homolog D (RBOHD). NADPH oxidase is activated by BIK1 and calcium-dependent proteins kinases (CDPKs). RBOHD could be regulated by Ca^{2+} binding to the N-terminal EF-hand motifs of the protein (Bigeard, 2015) and originates the superoxide anion (O^{2-}), in the apoplast. The superoxide anion is dismutated to hydrogen peroxide (H_2O_2) by superoxide dismutase which transfers across membrane passively or trough water channel. The immune response involves the secretion of defense-related proteins (PR), such as PR1 proteins, with antioomycete and antifungal action, and PR2 which are β-1,3-glucanases. PR proteins show strong antifungal and antimicrobial activity and some of them inhibit spore germination, by breaking down the structure of cell walls.

A second class of signalling compounds, jasmonic acid (JA), salicylic acid (SA) and ethylene (ET), are produced as endogenous signalling molecules that elicit pathogen protection process. Jasmonic acid (JA) and ethylene (ET)-mediated signalling dictate the synthesis of the defensin, which is more commonly associated with resistance to necrotrophs and response to wounding, herbivores, general elicitors and non-host pathogens.

SA is synthesised from chorismate, which resulted from shikimate

pathway, by the enzyme isochorismate synthase. The pathogen infection induces the synthesis of methyl salicylate (MeSA), a volatile ester, normally absent in plants. MeSA is synthesised by SA carboxyl methyltransferase and operates as a volatile signalling molecule. Tipically, SA is synthesized in the plants when the infection is caused by a biotrophic pathogen and induces the hypersensitive response that is followed by Systemic Acquired Resistance (SAR). In fact SA occurs in the phloem whereby it diffuses out in the entire plant. This leads to the activation of numerous effector genes. Moreover SA induces the synthesis of pathogenesis-related (PR) proteins.

Phase 2.

Some pathogens, including oomycetes, have the capacity to overcome the PTI.

The oomycetes are characterized by two different kinds of effectors: extracellular and intracellular elicitors (Jones and Dangl, 2006). The first effector class represents an evolved mechanism for protection against PR proteins, the hydrolytic enzymes secreted by the plants. The oomycetes cytoplasmic effectors possess a conserved domain featuring the motif RXLR and a C-terminal domains associated with virulence function.

The secreted oomycetes effectors interfere with PCD, PAMP-triggered ROS production and callose deposition leading to effector-triggered susceptibility (ETS).

Phase 3

The effector proteins (AVR) of the pathogen are recognized by R proteins of the plants, which activate the effector-triggered immunity (ETI), resulting in the HR (Kamoun *et al.,* 1999).

The intracellular elicitors are responsible for HR, triggered by a gene-for-gene interaction, in the host resulting in the cascade of reactions involved in the defence system.

Defence responses in the hosts are controlled by resistant genes (*R* genes) which are activated in presence of pathogen signals (the elicitors). Disease resistance is controlled by *R* genes, which encode for receptors of the plant and *Avr* genes that encode for effectors of the pathogen and control the virulence in a susceptible host. According to the gene-for-gene-interaction, if receptors of the host recognize specifity elicitors of the pathogen, resistance occurs and leads to the HR at the infection site. ETI is an accelerated PTI response

The receptors of the host plant are proteins which contain a functional domains consisting of nucleotide binding site (NBS) and a leucine rich repeat region (LRR). Numerous genetic studies have shown that the LRR domain controls specific recognition.

The basic principles of this model are: an effector acting as a virulence factor has a target(s) in the host; by manipulating or altering this target(s), the effector contributes to the pathogen success in susceptible host genotypes; effector perturbation of a host target generates a 'pathogen-induced modified-self' molecular pattern, which activates the corresponding NB-LRR protein, leading to ETI. The consequences are that: multiple effectors could evolve independently to manipulate the same

host target; more than one NB-LRR protein associated with a target of multiple effectors could evolve. NB-LRRs would be activated by recognition of different modified-self patterns produced on the same target by the action of the effectors (Jones and Dangl, 2006).

1.7 SOURCES OF RESISTANCE IN GRAPEVINE

Host-pathogen interactions are influenced by ecological and genetic factors which induce a co-evolving interaction.
Natural sources of disease resistance are found in geographic regions where pathogens and host plants co-evolved.

Some North-American varieties are partially or totally resistant to the pathogen and are used for grapevine breeding for their valuable source of resistant genes, whereas all *V. vinifera* varieties, the European grapevine, are homozygous recessive for resistence genes.

The American species, *V. labrusca* L.*, V. riparia* Mich.*, V. cinerea* Enghelm.*, V. aestivalis* Mich.*, V. rupestris* Scheele*, V. berlandieri* Pl.*, V. lincecumii* Buckley and *Muscadinia rotundifolia* Michx. are characterized by different level of resistence.

The quantitative trait loci (QTLs) that have been identified as major factors on downy mildew resistance are: *Rpv1* and *Rpv2* derived from *Muscadinia rotundifolia* (Merdinoglu *et al.,* 2003; Peressotti *et al.,* 2010); *Rpv3* originated from *V. rupestris* (Di Gaspero *et al.,* 2012); and *Rpv 8, Rpv10* and *Rpv12* derived from the Asian grapevine, *V. amurensis* (Blasi *et al.,* 2011).

Weak QTLs associated with minor effects have also been identified to bring a low of partial resistance to the pathogen: *Rpv4* (Welter *et al.,* 2007) *Rpv5* and *Rpv6* found in *V. riparia* (Marguerit *et al.,* 2009; Marino *et al.,* 2003), *Rpv7* (Bellin *et al.,*2009), *Rpv9* and *Rpv13* (Moreira *et al.,* 2011), *Rpv11* (Fischer *et al.,* 2004).

Rpv1 is located on chromosome 12 of wild American species, *rotundifolia* and *V. riparia*, and encodes for NBS-LRR proteins (Wiedemann-Merdinoglu *et al.* 2006; Feechan, 2013) and it is genetically associated to *Run1*, a locus conferring powdery mildew resistance (Merdinoglu *et al.,* 2003). A QTL in the same region was identified in *V. riparia*, which showed the reduction of sporangia released per unit of leaf area (Marguerit *et al.,* 2009).

Rpv2 is located on the distal part of chromosome 18 in *M. rotundifolia* (Wiedemann *et al.,* 2006).

The *Rpv3* locus, first reported in cv 'Regent', controls grapevine resistance to *P. viticola* and it is the major determinant of resistance. The resistance (*R*) genes, that encode TIR-NB-LRR and LRR-kinase receptor-like proteins, may occur at the *Rpv3* locus, which is situated in the lower arm of chromosome 18 in *V. riparia, V. labrusca* and *V. rupestris.* The resistance is based on hypersensitive response (HR) and the class of genes clustered at the *Rpv3* locus are NBS-LRRs: this implies that downy mildew resistance inherited by 'Bianca' from North American varieties is race specific (Bellin *et al.,* 2009). Moreover the co-localization of the genes that encode for TIR-NB-LRR with *Rpv3* could hint a putative functional role (Welter *et al.,* 2007).

A resistant *Rpv3*$^{+}$ haplotype has been introgressed from wild ancestors,

most likely an accession of the Midwestern American species *V. rupestris*, into *V. vinifera*-like descendants of the 'Villard Blanc' lineage (Casagrande *et al.*, 2011). *Rpv3* is associated with the localized hypersensitive response (HR), which occurs immediately after infection and it is correlated with a significant reduction of mycelial growth in the host tissues and a limitation of sporulation.

In resistant varieties 'Regent' and 'Bianca', possess the *Rpv3* gene in heterozygous state. This causes a partial resistance to downy mildew that has been overcome by pathogen isolates, that evaded the recognition or suppress effector-triggered immunity (Peressotti, 2010; Delmotte, 2013).

Rpv8, located on chromosome 14, is the first QTL conferring resistance to *P. viticola* discovered in an Asian *Vitis* species and confers total resistance to downy mildew in the *V. amurensis* 'Ruprecht' cultivar from which it originates (Blasi *et al.,* 2011). Probably *Rpv8* is a member of the NBS-LRR class of disease resistance genes (Blasi *et al.,* 2011).

The introgression of *P. viticola* resistance *Rpv10* from *V. amurensis* (Schwander *et al.,* 2011) in *V. vinifera* led to the selection of different cultivars, among which there is Solaris. *Rpv10* is associated with necrosis formation, callose deposition, and stilbene accumulation (Zini *et al.*, 2015). A stress activated, ethylene-responsive transcription factor and an ankyrin-like protein are tightly linked to *Rpv10*: ethylene is an endogenous plant hormone that influences the plant response and acrivates the proteins containing ankyrin repeats, like the NPR1 (Nonexpressor of *PR* genes 1) that plays a key role in the salicylic acid pathway that leads to systemic acquired resistance (SAR) (Schwander *et al.,* 2011).

The *Rpv12* locus coincides with a cluster of CC-NB-LRR genes, a less

dynamic subclass, localised on chromose 14 and derived from *V. amurensis,* that is associated with a localised HR (Venuti *et al.,* 2013).

Except for *Rpv2* and*Rpv8*, all the afore mentioned loci have been used in grapevine breeding programs.

A minor QTL for downy mildew resistance was identified by Welter *et al.* (2007) on chromosome 4. The presence of a major QTL accompanied by minor QTLs (with minor effects) appears to be a common phenomenon in plant genetics of resistance (Welter *et al.,* 2007).

In a cross between *V. vinifera* 'Cabernet Sauvignon' and *V. riparia* 'Gloire de Montpellier' two resistance loci were identified on chromosome 9 and 12 (Marguerit *et al.,* 2009) and were designated *Rpv5* and *Rpv6*. The minor QTLs explained a reducted percentage of the observed phenotypic variance (Marguerit *et al.,* 2009).

A minor QTL on chromosome 7 (*Rpv7*) of 'Bianca' was found in a cross between 'Chardonnay' and 'Bianca' by Bellin *et al.* (2009). The *Rpv7* was consistently scored for the extent of pathogen growth and sporulation, which explained a limited part of the residual phenotypic variance (Bellin *et al.,* 2009).

Moreira *et al.* (2011) reported a resistance locus (*Rpv9*) on chromosome 7 using a cross between *V. vinifera* 'Moscato Bianco' and a *V. riparia* that showed resistance to downy mildew, explaining a low percentage of phenotypic variance observed and *Rpv13* on chromosome 12 in a *V. vinifera* 'Moscato Bianco' x *V. riparia*, mapped close to *Rpv1*.

Rpv11 was identified by Fischer *et al.* (2004) on chromosome 5 of 'Regent'. This locus was also reported in 'Chardonnay' (Bellin *et al.,* 2009).

1.8 Vitis vinifera

1.8.1 Caucasian varieties

Various analysis on the correlations between *V. vinifera sylvestris* and *vinifera* subspecies on samples collected from all Eurasian country confirmed the hypothesis of the southern area as domestication origin of grapevine (Myles *et al.,* 2011). Moreover investigations focused on "Grape's Fertile Triangle" revealed the close genetic relationship between local wild grapevines and varieties traditionally cultivated in southern Anatolia, Armenia and Georgia, confirming that the source of the Tigris and the Euphrates in the Taurus Mountains are the more probable areas in which collocating the first domestication of grapevine (Vouillamoz *et al.,* 2004). The Transcaucasian region, is the area comprised between the Black Sea and the Caspian Sea, which extends from Main Caucasus to the Iranian and Turkish borders, including republics of Georgia, Armenia and Azerbaijan, is a region characterized by a rich biodiversity and by the presence of numerous grapevine wild species, the ancestral forms of cultivated species (Vavilov, 1926; Negrul, 1946).

Some Georgian authors affirm that the oldest root of the word "wine" would be Kartvelianoγwine/ღვინო, a still used term in the modern Georgian language and it is an irrefutable evidence that the Georgia is the cradle of viticulture (Gamkrelidze *et al.*, 1990).

Georgia is rich in many specimens of *Vitis vinifera* ssp. s*ylvestris* still today which grow at high altitudes in a range between 20 and 1000 meters.

In this region the first stable settlement date back to VII millennium B.C.; archeological and paleobotanical evidences of this period have been found in sites of Shulaveri, located 30 Km apart from Tbilisi and belonging to period of Shulaveri-Shomu culture (6000-4000 b.C.). Among the evidences that have been found are: seeds of grapevine, which indicate unequivocally the cultivation of *Vitis vinifera* L. ssp. *vinifera*; a Dergi wine vase with an incision of a bunch; fragments of other crockery with depiced grapes and statues of fertility goddesses (Mc Govern, 2003); wine remains in a Shulaveri jar.

The viticulture in Georgia at the end of V and the beginning of IV millennium b.C. started as demonstrated by the remains of elaborate wine cellar found and "kvevri", huge clay vases, still containing grapeseeds and traces of wine obtained from different vine varieties. Omero and many other Greek authors song the praises of scented wines and sparkling of Colchis, that were exported throughout the Mediterranean basin.

Among the most ancient citations of the wine cultivation in Caucasus, there is that of Apollonio Rodio (295 b.C. – 215 b.C.); when Jason and the Argonauts arrived in Colchis, the current western Georgia, they found a wine fountain in the palace of Aieti and they rested in the shadow of grapevines. The Colchis is the most ancient area of grapevine cultivation. According to Herodotus (V c. B.C.) and Strabone (I c. B.C.) the wine-making prospered in Georgia: "bubbly and sweet, as honey, Colchis wine", is often mentioned in their letters. The history and myth are confirmed by evidences of grapeseeds in Ergeta (district of Zugdidi, VII-VI c. b.C.) and Gienos (area Ochamchire, VII-VI c. b.C.) belonging to *V. vinifera* ssp. *vinifera* and *V. vinifera* ssp. *sylvestris* (Rusishvili, 2010).

After the period of the Shulaveri culture, Mtkvari (Kura)-Araks started in southern Caucasus (4000-2000 B.C.). The period was characterized by a increased agricultural production and graziery, by metallurgy progress and by life quality improvement: the Badaani site shows signs of soft wheat, Persian wheat, called Dika in Georgian, barley cultivation in addition to signs of grapevine cultivation. In the early part of the II millennium B.C. the Trialeti culture spread, reaching its maximum in 1500 b.C. in eastern Georgia. Large burial mounds ('Kurgans') found at the site of Trialeti itself and other sites of the period have yielded marvelously ornate gold and silver goblets, often depicting drinking scenes or ceremonies, highlighting the importance of the vine to this culture (Maghradze *et al.,*2016).

The more fertile period of Caucasian viticulture is localised in the first few centuries A.D., also due to the influence of Christianity; the importance of the grapevine cultivation and of the oenology for Georgia was identified by the Christianity symbolic figurehead of the country: Saint Nino, the nun which brought this religion in Georgia from Cappadocia in the IV century A.D.

The Saint converted the King of Iberia using a cross made of vine shoots, plant and symbol which has been resurfaced in frescos and in low reliefs in many monasteries of the territory, religious places. The rebirth of the viticulture in this region starts on the end of 1800; in 1801, when Georgia was included in the Russian empire, new wine cellars were constructed or the oldest renovated and Georgia has been exploited by the Union of Soviet Socialist Republics as "wine cellar of the empire".

At the dissolution of the USSR, Georgia joined the Confederation of Independent States but later withdrew. The disputes with Moscow lead to a trade embargo by the government of CSI in 2006, which involved in particular the wine; this embargo brought positive and negative changes in the wine market: the producers had necessarily to improve the quality of wines to sell the product in other markets but, on the other hand, some wine cellars failed. Ukraine is, at the moment, the largest importer of Georgian wine, followed by Kazakhstan and Belarus. Other states, which acquire wine from Georgia, are United States, China, Baltic countries and Azerbaijan.

Georgia is a state of 69700 km^2 situated in the Southern Caucasus, in the western of Transcaucasus and it is bordered to the north by the Greater Caucasus mountain range, which has a very important role of protection from the cool air masses coming from north, to the south by Lesser Caucasus, which partially mitigates the influence of the dry-hot air coming from south, while to the west is bathed by Black Sea.

With the exception of the fertile plain of Kolkheti, Georgia is largely mountainous and more than one third is covered by forest and undergrowth. The wide variety of landscapes stretches from the subtropical banks of Black Sea to the Caucasian snowy crest. The Likhi mountains divide the country into two parts from north to south, Eastern and Western Georgia. Considering the country size, the climate is extremely diversified: it is characterized by a damp subtropical climate to the west, maritime, while to the east there is a variability depending on different altitudes.

During the same season, the climate can range from damp subtropical to alpine, and on the top of mountains can be found snow and ice during all year. In the central part, winter is rigid, with low temperature ranging from -12°C to -14°C. Precipitation, in view of the complex orography, varies from 300 mm annually in the eastern part to 2000 mm in the western; the Black Sea represents a source of hot and humid air.

In Transcaucasus two grapevine domestication areas can be identified: Alazani, comprising eastern Georgia and neighbouring territories of Azerbaijan and Armenia; and Colchis, comprising western Georgia and Black Sea coast.

The largest areas of grapevine cultivation in Georgia are represented by Kakheti regions (52% of vineyards), Imereti (22%), Kartli (11%), Racha-Lechkumi (4-5%), Guria, Samegrelo and Adjara (2-3%) (Fig. 1.7); generally in each region specific varieties are cultivated, while for the production of wine two technologies have been developed: Kakhetian and Imeretian methods, getting their names from the regions.

Kakhezia, in east, is the centre of Georgian classic viticulture and consist of a plateau place with a height comprised between 400 and 800 m, intersected by Alazani and Iori rivers, with a temperate climate. The main varieties cultivated are Saperavi, Rkatsiteli, Mtzvane of Khaketi, Kisi and Khikhvi. The grapes are poured in wood receptacles containing ferns on the bottom that allow the must to flow in characteristic terracotta vases, kvevris, where the fermentation takes place. Each vase, which contains about 1500 L, is buried to the neck and the fermentation continues for 7-10 days. Kvevris are then hermetically sealed with a big stone and covered

by a layer of soil. For red wines the marcs are removed, while for white wines they remain in the vases.

In January-February the wine is moved to cleaner kvevris. The vinification gives to the wine a characteristic bouquet, strongly scented and an intense colour.

The Imereti region, corresponding to legendary Colchis area, is located to the west; the climate is very humid. The local white vine varieties are Tsolikouri, Tsiska, Krakhuna, Goruli, Mzvane and the red varieties Otskhanuri, Sapere and Saperavi. The white sparkling wines obtained by Tsiska grapes are particularly appreciated. The wine production method consists of putting only 5-10% of stalks, seeds, marcs, producing a more acid wine and characterized by a lower alcohol level than wines produced in Kakhezia.

In the last years, several Georgian native varieties were inserted in breeding programs in Georgia as in other foreign countries. As a result, 193 new varieties were bred in 15 countries, with the contribution of 13 Georgian native varieties. Vine cultivation and pest diseases in the past had not affected the germplasm structure of Georgian cultivated varieties as much as in other countries. This makes the country viticulture challenging when thinking about the possibilities offered by breeding for quality and/or resistance (Imazio *et al.,* 2013)

Figure 1.7: Map of Georgia with the names of the main viticultural aereas (Imazio *et al.,* 2013).

2. AIMS OF THE WORK

Grapevine (*Vitis* spp.) is one of the most extensively cultivated plants with a worldwide economic importance. Grapevine is susceptible to different pathogens that are responsible for serious crop losses epidemics among which are: *Erysiphe necator* Schwein., the agent of powdery mildew; *Botrytis cinerea* Pers., the agent of grey mould; and *P. viticola*, which causes downy mildew. Downy mildew can be considered the most severe disease in the wine-growing areas characterized by abundant rainfalls during spring-summer and relatively mild temperatures. *P. viticola* can severely reduce both the quality and the quantity of the yield. The north of Italy is the area most affected by the disease, but the pathogen can have a significant negative impact on grapevine also in the central and southern regions. The potential harmful of the pathogen, combined with a low efficacy of the agronomic practices in contrasting *P. viticola*, always made the use of chemical control necessary for the defence against downy mildew. The European Directive 2009/128/EC establishes a framework for Community action to achieve the sustainable use of pesticides. One of the key features of the Directive is that each Member State should develop and adopt its National Action Plan and set up quantitative objectives, targets, measures and timetables to reduce risks and impacts of pesticide use on human health and the environment and to encourage the development and introduction of integrated pest management and of alternative approaches or techniques in order to reduce dependency on the use of pesticides. Other provisions include compulsory testing of application equipment, training and certification of all professional users, distributors and advisors; a ban (subject to derogations) on aerial spraying; special measures to protect the aquatic environment, public spaces and

conservation areas; minimizing the risks to human health and the environment through handling, storage and disposal.

A real possibility of limiting the number of treatments, which in critical years can be higher than ten, lies in the cultivation of grapevine varieties resistant to the pathogen. The resistant varieties are usually obtained by crossing varieties with good qualitative characteristics and cultivars characterized with resistance genes towards the pathogen, that originated from areas in which the host coevolved with the pathogen (Belli, 2006). The host-pathogen coevolution leads to the selection of hosts that are able to resist to the infection through the selection pressure exerted by the pathogen. The resistance of the plant to the pathogen imposes, in turn, a selection pressure on the populations of the pathogen. The contemporary viticulture modifies this equilibrium, in particular because the selection of the cultivars promotes the genotypes that are more suitable for grape quality and production than for resistance to pathogens, favoring consequently the onset of severe disease epidemics. Since the arrival of *P. viticola* in Europe, numerous genetic improvement programs have been undertaken to introduce the resistant genes of the American species, co-evolved with the pathogen, in *V. vinifera*, the European grapevine, to obtain resistant varieties. The first hybrids obtained, however, produced low quality grapes due to the unpleasant foxy aromas conferred by the American vines. Until few years ago, the possibility that *V. vinifera* varieties might be characterized by the capacity to limit the pathogen infections was not considered. However, finding source of resistance in European grapevine could contribute to obtain qualitative interesting varieties. The resistant genes should be investigated in grapevine populations characterized by a high genetic variability, such as of

Caucasian origin. The Caucasus is characterized by a rich biodiversity and for the presence of numerous wild grapevine species, *Vitis vinifera* subsp. *sylvestris*, which represents the ancestral form of the cultivated vine, named *V. vinifera* subsp. *vinifera* (Vavilov, 1926; Negrul, 1946). In a preliminary screening activity carried out at the DiSAA Department of the University of Milan, on Caucasian varieties of *V. vinifera*, some accessions proved to be resistant to *P. viticola* both in experimental inoculations and in the field. The cv Mgaloblishvili N., hailing from the Imereti province, located in western Georgia, was characterized by the most stable behaviour (Toffolatti *et al*., 2016). The variety is classified as Proles *pontica* subproles *georgica* Negr. and no intravarietal phenotypic variations have been revealed so far. During 2012, in the experimental vineyard located in the Research Centre of Riccagioia (Torrazza Coste, PV), the resistant Caucasian accession was crossed with Pinot noir (susceptible variety) to screen the progeny for the susceptibility level towards *P. viticola*. Mgaloblishvili progenies were obtained also by self pollination and open pollination. A parallel genetic investigation, aiming at characterizing the genes which control the resistant characters and developing a genetic map of Mgaloblishvili, is in progress at the Research Centre of Edmund Mach located in S. Michele all'Adige (TN).

Some pathogens are characterized by a high genetic variability, and consequently a great evolutionary potential that confers the ability to break down the resistance mechanism of the plant (McDonald, 2002). *P. viticola* has a high asexual sporulation efficiency, a polycyclic behavior and sexual reproduction through the formation of oospores and proved to possess a high evolutionary potential; (Peressotti *et al.,* 2010; Toffolatti, 2012). The development of the pathogen in the host tissues could be modulated by the presence of resistant gene(s) of the plant. Comparing the

growth of *P. viticola* in Bianca variety, characterized by the resistant gene *Rpv3*, with the development of the pathogen in Mgaloblishvili, could suggest the kind of resistance of Caucasian accession. The behaviour of the plants could depend on the aggressiveness level of the pathogen. 'Aggressiveness' is define as the relative ability of a plant pathogen to colonize and cause damage to plants (Pariaud, 2009). The durability of resistance genes of the plants depends more on the genetic characteristics of the pathogen which control the aggressiveness level, than to the nature of the resistance gene (McDonald, 2002). The level of aggressiveness can be evaluated by estimating the disease severity (percentage index of infection) and the sporulation rate (number of produced spores) (Pariaud, 2009). Several studies have suggested that aggressiveness components are controlled by a genetic basis with a polygenic determination. In Italy no informations are present on the genetic variability of the populations of *P. viticola* therefore it is not possible at the moment to evaluate the genetic relationships and differences between the pathogen populations which could influence the aggressiveness level.

The aims of the present work are:

1) to find possible source of resistance in *V. vinifera* by screening for resistance to *P. viticola* the DiSAA collection of Caucasian and Iranian varieties, wild and cultivated, by experimental inoculation and field evaluation;
2) to characterize the resistant phenotype of the progenies obtained from cv Mgaloblishvili in order to evaluate the segregation of genes involved in resistance control;
3) to characterize the interaction between *P. viticola* and Mgaloblishvili by histological analysis at different infection stages;

4) to analyze the aggressiveness levels of different *P. viticola* populations collected from northern Italian vineyards;
5) to investigate the genetic diversity of *P. viticola* populations collected from different Italian regions by microsatellite analysis at the Research Centre INRA Bordeaux-Aquitaine.

3. MATERIALS AND METHODS

3.1 PLANT MATERIAL

3.1.1 In field

The 94 Georgian grapevine varieties used in this study (Table 3.1) are grown in a collection vineyard established in 2006 at the Regional Research Station of Riccagioia located in Lombardy at Torrazza Coste (PV) region of northern Italy. The site is located in the Oltrepò pavese viticultural area (long. *9°05',* lat. *44°58',*elevation 144 m a.s.l.) on a hilly terrace with a slight east exposition with a typical clay soil *(Udic Paleustalfs fine silly, mixed, superaclive, mesic* following the USDA soil taxonomy by Soil Survey Staff, 1999). The initial plant propagation material was taken from the grapevine collection of Georgian ancient cultivars established in locally named as 'Dighomi' located closed to the Georgia capital Tbilisi in 1967/1968 and belonging to the Agricultural University of Georgia.

The trichome density of the accessions was determined on the lower side of leaves following the OIV method code 84 (2001).

Table 3.1: List of Georgian varieties in relation to their region of origin, berry colour and density of the hairs between the veins on the lower side of the leaf.

ID	Name of variety	Region of origin	Berry colour*	Hair density**
L21A	Okroula	Kakheti	B	3
L21B	Tsnoris Tetra	Kakheti	B	3
L21C	Kurkena	Kakheti	B	3
L21D	Akhmetis Shavi	Kakheti	N	3
L21E	Saperavi Grdzelmtevana	Kakheti	N	3
L21F	Zakatalis Tsiteli	Kakheti	N	3

ID	Name of variety	Region of origin	Berry colour*	Hair density**
L22A	Mgaloblishvili	Imereti	N	7
L22B	Marguli Sapere	Imereti	N	5
L22C	Gabekhouri Tsiteli	Imereti	N	7
L22D	Endeladzis Shavi	Imereti	N	3
L22E	Mtsvane Onidan	Ratcha	B	5
L22F	Usakhelouri	Ratcha	N	3
L23A	Khushia Shavi	Imereti, Guria	N	7
L23B	Orona	Guria	N	5
L23C	Ikaltos Tsiteli	Kakheti	N	7
L23D	Okhtoura	Kakheti	N	7
L23E	Kistauris Saghvine	Kakhuri	N	5
L23F	Vertkvichalis Shavi	Imereti	N	5
L24A	Satsuravi	Adjara	N	7
L24B	Khrogi	Ratcha	N	3
L24C	Zakatalis Tetri	Kakheti	B	5
L24D	Mtsvivani Mskhvilmartsvala	Kakheti	B	3
L24E	Jghia	Kakheti	N	5
L24F	Chinuri	Kartli	B	3
M21A	Ghvinis Tsiteli	Kakheti	N	3
M21B	Kharistvala Shavi	Kakheti	N	3
M21C	Tkupkvirta	Kakheti	N	3
M21D	BudeshuriTsiteli	Kakheti	N	3
M21E	Buera	Kakheti	B	3
M21F	Goruli Mtsvane	Kartli	B	7
M22A	Zerdagi	Samegrelo	N	7
M22B	Paneshi	Samegrelo	N	5
M22C	Chkhucheshi	Samegrelo	B	3
M22D	Chkhaveri	Guria	N	7
M22E	Kamuri Shavi	Guria	N	5
M22F	Jani Bakhvis	Guria	N	5
M23A	Tkbili Kurdzeni	Kakheti	N	7
M23B	Kuprashviliseuli	Imereti	N	7
M23C	Dzelshavi Obchuri	Imereti	N	3
M23D	Mirzaanuli	Kakheti	B	3
M23E	Chkhikoura	Imereti	B	3

ID	Name of variety	Region of origin	Berry colour*	Hair density**
M23F	Kapistoni Tetri	Imereti	B	3
M24A	Asuretuli Shavi	Kartli	N	3
M24B	Tavkara	Kakheti	N	7
M24C	Argvetula	Imereti	N	7
M24D	*Vitis x labruscana*	Georgia	N	7
M24E	Ananura	Kartli	N	3
M24F	Tchvitiluri	Samegrelo	B	7
N21A	Gorula	Kartli	B	3
N21B	Tita Kartlis	Kartli	B	3
N21C	Adreuli Tkhelkana	Kartli	N	5
N21D	Shavkapito	Kartli	N	5
N21E	Ghrubela Kartlis	Kartli	B	3
N21F	Buza	Kartli	N	5
N22A	Otskhanuri Sapere	Imereti	N	7
N22B	Orbeluri Ojaleshi	Lechkhumi	N	5
N22C	Aleksandrouli	Ratcha	N	5
N22D	Rkatsiteli	Kakheti	B	3
N22E	Kumsmtevana	Kakheti	B	3
N22F	Sirgula	Kakheti	B	3
N23A	Tsolikouri Mtsvivani	Imereti	B	5
N23B	Bazaleturi	Imereti	B	5
N23C	Tsirkvalis Tetri	Imereti	B	5
N23D	Vertkvichalis Tetri	Imereti	B	5
N23E	Imeruli Shavi	Imereti	N	5
N23F	Adanasuri	Imereti	N	5
N24A	Ojaleshi	Samegrelo	N	5
N24B	Aladasturi	Guria	N	7
N24C	Tchumuta	Guria	N	7
N24D	Khushia Shavi	Imereti, Guria	N	7
N24E	Badagi	Guria	N	7
N24F	Acharuli Tetri	Adjara	B	-
O21A	Tamaris Vazi	Kartli	N	3
O21B	Saperavi Atenis	Kakheti	N	5
O21C	TkvlapaShavi	Imereti	N	5
O21D	Tavkveri	Kartli	N	3

ID	Name of variety	Region of origin	Berry colour*	Hair density**
O21E	Shavtsitska	Imereti	B	5
O21F	Dondghlabi	Imereti	B	5
O22A	Sapena	Kakheti	B	5
O22B	Ubakluri	Kakheti	B	5
O22C	Rkatsiteli Vardisperi	Kakheti	Rs	3
O22D	Tsqobila	Kakheti	N	5
O22E	Danakharuli	Kartli	B	3
O22F	Chitiskvertskha Meskhuri	Kartli	N	5
O23A	Maghlari Tvrina	Imereti	N	5
O23B	Rko Shavi	Imereti	N	5
O23C	Dziganidzis Shavi	Imereti	N	7
O23D	Didshavi	Imereti	N	7
O23E	Kvelouri	Imereti	B	7
O23F	Samarkhi	Guria	B	7
O24A	Avasirkhva	Abkhazeti	N	3
O24B	Kachichi	Samegrelo	N	5
O24C	Shonuri	Samegrelo	N	7
O24D	Aspindzura	Kartli	N	5

*N: noir (black); B: blanc (white); Rs: rose;

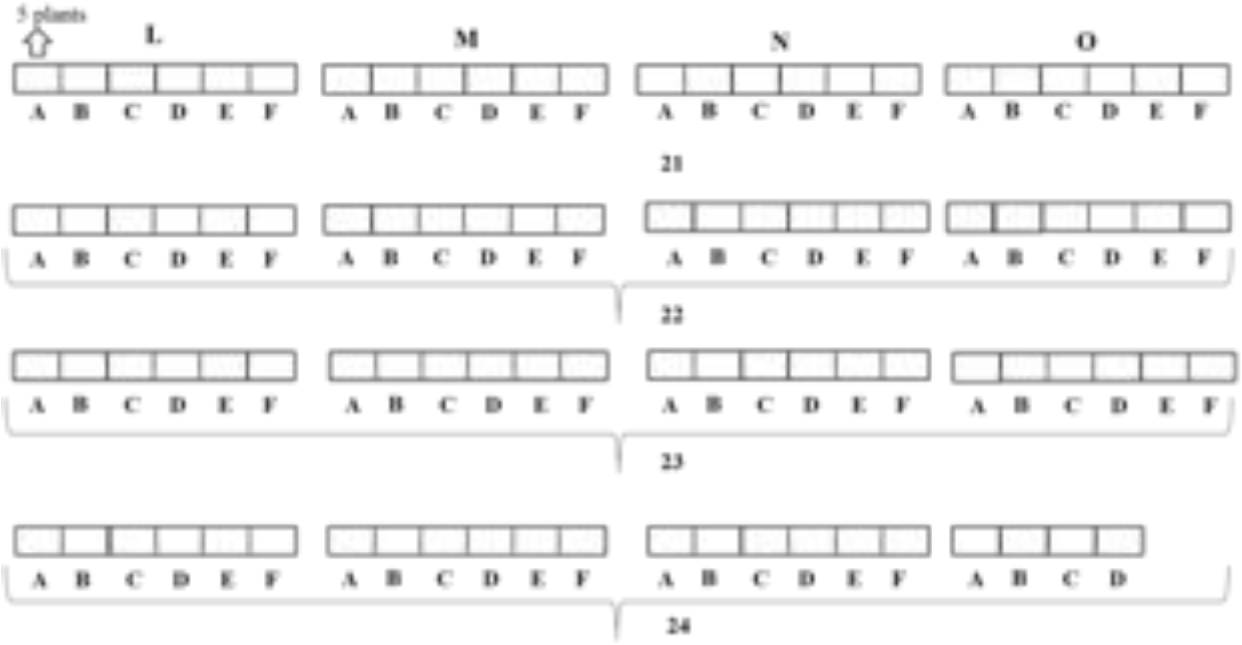
** 3: low; 5: medium; 7: high

The investigated accessions are all *V. vinifera* varieties native to Georgia (South Caucasus region) apart from one: a *Vitis x labruscana* L.H. Bailey (accession M24D), belonging to the Georgian *Vitis* germplasm, was included in this survey as resistant control accession. The downy mildew incidence was estimated also in an untreated plot of *V. vinifera* 'Croatina N', fully susceptible to *P. viticola*, placed immediately nearby. The 'Croatina N' plot has the same characteristics of the Georgian varieties and consists of three rows 50 m long. Plants were grafted on 1103 Paulsen (*V. berlandieri* x *V. rupestris*) rootstock, spaced at 2.5m (inter-row) x 1 m (intra-row), trained to the Guyot system at a density of 4,000 plants/ha with a two-bud spur and a 10- to 12-bud cane. The inter-row soil was kept

weed free by two yearly glyphosate herbicide treatments. Each accessions consists of five plants per variety.

The vineyard was divided in four row, from 21 to 24, four parcels, indicated by the letters from L to O and six inter poles from A to F (Figure 3.1). The codes of the plants derived from their position in vineyard.

Figure 3.1: Scheme of the plots in vineyard



3.1.2 In screenhouse

Four plants of Mgaloblishvili (L22A), 148 Caucasian and Iranian *V. vinifera* subsp. *sativa* and 35 Caucasian *V. vinifera* subsp. *sylvestris* plants were cultivated in pots (20 cm diameter) at University of Milan screenhouse, located in Tavazzano con Villavesco (LO.

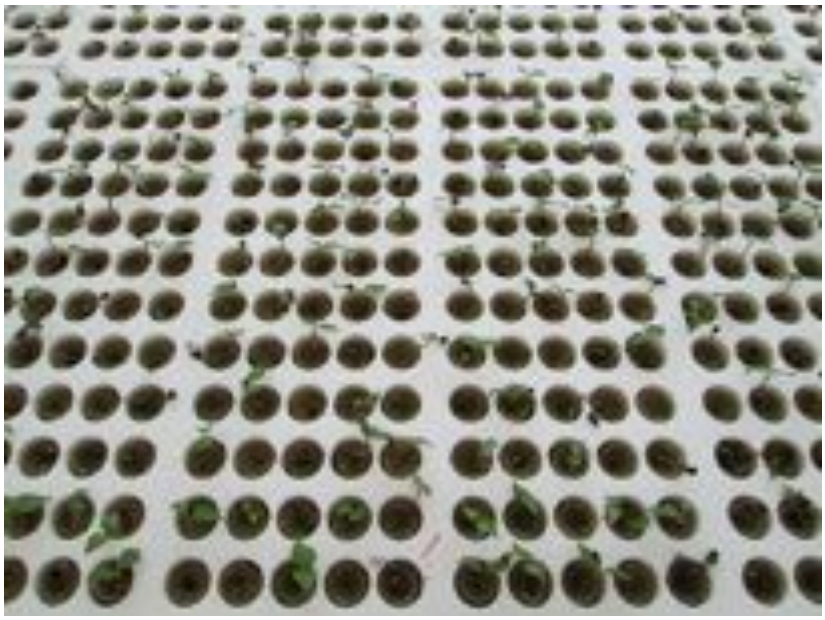
In spring 2012, different progenies were obtained from cv Mgaloblishvili: of the 272 total individuals obtained, 23 originated by pollinating cv Mgaloblishvili flowers deprived of styles with Pinot noir pollen; 158

derived from self pollination of Mgaloblishvili, often enclosing the inflorescences in paper bags; and 91 individuals originated by open pollination with pollen freely circulating in vineyard.

Figure 3.2: Inflorescences covered by paper bags after pollination in the field.

During 2012, at maturation, bunches were harvested to collect grape seeds. After a vernalization consisting period of 2 months at 5°C, the seeds were placed to germinate in plate of polystyrene cups Grodan at 20 to 25 °C (Figure 3.2).

Figure 3.3: Grapevine seedlings growing in Grodan cups



The seedlings were transplanted into individual pots (8 centimeters diameter) containing commercial peaty substrate mixed with sand and soil, in screenhouse.

The seedlings were regularly irrigated and it was not necessary to administer mineral fertilizers.

The Georgian and Iranian *V. vinifera* subsp. *vinifera* accessions screened for resistance to *P. viticola* are listed in Table 3.2 and 3.3. The Caucasian accessions of *V. vinifera* subsp. *sylvestris* are listed in table 3.4.

The plants were not treated with fungicides active against *P. viticola*.

Table 3.2: List of Georgian Caucasian *V. vinifera* subsp. *vinifera*

Accession ID	Name of variety
F01C01	Tsulukidzis tetra
F01C02	Tita kartlis
F01C05	Chitistvala kakhuri
F01C06	Tchetchi peshi

Accession ID	Name of variety
F01C07	Tskhvedianis tetra
F01C08	Kakhis tetra
F01C09	Mskhviltvala tetri
F01C10	Tsirkvalis tetri
F01C12	Klarjuli
F01C16	Gomis tetri
F01C17	Gorula
F01C18	Mtsvane kakhuri
F01C19	Rkatsiteli
F01C20	Brola
F01C22	Atcharuli tetri
F02C03	Djvari
F02C04	Kumsmtevana
F02C05	Tsitska sachkheris
F02C06	Tsitska
F02C07	Chekobali
F02C11	Chekhardani
F02C12	Krakhuna
F02C13	Tsolikouri
F02C14	Sirgula
F02C15	Sakmevela
F02C19	Tsolikouri mtsvivani
F02C20	Bazaleturi
F02C21	Vertqvitchalis tetri
F02C22	Muradouli
F03C04	Khapshira
F03C05	Adreula tkhelkana
F03C06	Budeshuri tetri
F03C07	Bzvanura
F03C09	Aghbij
F03C10	Kharistvala tetri
F03C11	Almura tetri
F03C12	Kamuri tetri
F03C13	Kumsi tetri
Accession ID	Name of variety
F04C21	Tvaldamtsvri seuli

F04C22	Ghrubela kartlis
F05C04	Argvetuli sapere
F05C07	Vazisubnis tsiteli
F05C08	Portoka
F05C09	Tchumuta
F05C11	Saperavi
F05C14	Maghlarishavi
F05C17	Mudjuretuli
F05C18	Shavtkhila
F05C20	Mtsvane avrekhi
F06C01	Otskhanuri sapere
F06C04	Aladasturi
F06C05	Ktsia
F06C06	Shavkapito
F06C07	Kharistvala shavi
F06C08	Aleksandrouli
F06C09	Amotkhvij
F06C10	Amlakhu
F06C11	Ghvanura
F06C13	Imeruli schavi
F06C16	Abshiluri/avshiluri
F06C17	Shaviqurdzeni
F06C20	Tavkveri patalaanteuli
F07C01	Mekrenchkhi
F07C03	Adanasuri
F07C04	Djineshi
F07C06	Badagi
F07C10	Djani
F07C17	Mamukasvazi
F07C19	Noshrio
F08C02	Opoura
F08C03	Orbeluri odjaleshi
F08C07	Mtevandidi
F08C10	Tsitelouri
F08C11	Tchodi salkhinosi
Accession ID	Name of variety
F08C12	Batomura

F08C15	Samtchatcha
F08C17	Dzveli aleksandrouli
F08C18	Odjaleshi
F08C19	Aladasturi
F08C21	Khupishij
F08C22	Akomshtali
F09C05	Skhilatubani
F09C06	Odjaleshi
F10C07	Seura
F10C15	Mrgvalivardi speriqurdzeni
F11C02	Kornistvala
F11C03	Charitvala sciavi
F11C04	Saperavi mskhvilmartsvala
F11C05	Ojaleshi
F11C07	Matchkvaturi tskhakaiasi
F11C09	Jatchvadziseuli
F11C11	Mugiuretuli
F11C12	Kvira
F11C13	Orona shemokmedis
F11C14	Uchakardani
F11C15	Tkiskurdzeni
F11C16	Endela dziseuli
F11C18	Saperavi budeshuri seburi
F11C22	Kashmis saperavi
F12C19	Alexandrouli
F12C20	Saperavi clone 359
F12C22	Kashmis saperavi
F15C08	Budescuri tetri
F15C09	Chicvi
F15C10	Kochtura
F15C11	Almura tetri
F15C12	Kamuri tetri
F15C13	Chekardani
F15C15	Kumsi tetri
F15C16	Mtsvane kakhuri (clone 12)
Accession ID	Name of variety
F15C18	Kapistoni tsitsiliani

F15C19	Kumsi tetri
F15C21	Kishuri tetri
F16C07	Borkara
F16C08	Mkhargrdzeli
F16C09	Gldanula (gorula)
F16C10	Chitistvala kakhuri (bobduri)
F16C11	Tavtsitela
F16C12	Supris tetri
F16C13	Pashaniki
F16C14	Kharistvala meskuri
F16C15	Mskhviltvala tetri
F16C16	Gorula clone
F16C17	Beglaris kurdzeni
F16C18	Andreuli tkhelkana
F16C19	Bua kurdzeni
F16C20	Sabatono
F16C21	Adreula tchelkana
F16C22	Mskhvili kurdzeni
1-14 B	Beglaris kurdzeni
1-17 A	Rkatsiteli tsiteli
12 AR 12	Unknown
1-37 B	Kharistvala meskhuri
1-38 A	Gorula (clone)
1-7 B	Mskhvili Kurdzeni
2-13 A	Dzvelshavi
2-17 A	Dondghlabi Shavi
3-10 B	Almura Tetri
32-2	Borchalo
37-1	Institutis Grdzelmtevana
G-36	Unknown
SK	SaperaviKhashmis

Table 3.3: List of Iranian Caucasian *V. vinifera* subsp. *vinifera*

Accession ID	Name of variety
F26C01	Sabzangor
F26C02	Fakhri
F26C03	Chesmgave
F26C04	B603
F26C05	Yaghoyired
F26C06	Laal
F27C02	Sahebi
F27C03	Abak
F27C04	Sefiddaneh
F27C05	Chesmgave
F27C06	Shanei o Beidaneh
F27C07	Shastaroos
F27C08	Yaghoti white

Table 3.4: List of Caucasian Georgian *V. vinifera* subsp. *sylvestris* in relation to their region of origin.

Accession ID	Name of variety	Region of origin
13G007	Ninotsminda 12	Kakheti
13G009	Sartichala 04	Kakheti
13G012	Sartichala 08	Kakheti
13G013	Skra 01	Kartli
13G014	Naghomari 01	Lentekhi
13G027	Zubi 01	Lechkhumi
13G029	Tskhomareti 01	Lechkhumi
13G030	Larchvali 01	Lechkhumi
13G031	Lazgveria 01	Lechkhumi
13G032	Paldo 02	Kakheti
13G035	Mesamotse kvartali 02	Kakheti
13G036	Mesamotse kvartali 03	Kakheti
13G037	Baisubani 01	Kakheti
13G038	Kvetari 03	Kakheti
13G039	Kvetari 04	Kakheti
13G042	Kvetari 10(2)	Kakheti

Accession ID	Name of variety	Region of origin
13G047	Samebis seri 02	Kakheti
13G050	Samebis seri 08	Kakheti
13G052	Sabue 01	Kakheti
13G054	Sabue 03	Kakheti
13G056	Tushis tbebi 02	Kakheti
13G059	Shirikhevi 09	Kartli
13G060	Bagichala 04+05	Kartli
13G066	Bagichala 16	Kartli
13G069	Tedotsminda 04	Kartli
13G073	Tedotsminda 08	Kartli
13G075	Tedotsminda 10	Kartli
13G080	Tedotsminda 16	Kartli
13G085	Tedotsminda 21	Kartli
13G090	Unknown	Unknown
13G091	Lamiskhevi (enageti) 01	Kartli
13G093	Meneso 01	Kartli
13G097	Barisakho turning 01	Kartli
13G098	Barisakho turning 02	Kartli
13G101	Unknown	Unknown
13G103	Nakhiduri 06	Kartli
13G104	Nakhiduri 10	Kartli
13G105	Nakhiduri 11	Kartli
GEO W 104	Unknown	Unknown
GEO W 27	Unknown	Unknown
GEO W 31	Unknown	Unknown
GEO W 69	Unknown	Unknown
WF 10	Unknown	Unknown
WF 110/298	Unknown	Unknown
WFKTSIA 12	Unknown	Unknown
WFKTSIA 52	Unknown	Unknown

3.2 FIELD EVALUATION

The downy mildew incidence on the Georgian varieties cultivated in vineyard was assessed in July at BBCH 79 phenological phase (Lorenz *et al.*, 1994) for three consecutive grapevine growing seasons (2014, 2015, and 2016) by calculating the percentage of infected leaves and bunches (I%D) over the total.

100 leaves, randomly chosen, were visually inspected for the disease symptoms in relation with the total number of the leaves for each accession, to calculate the percentage of infected leaves. On bunches, it was counted the number of infected organs correlated with the total number of bunches for each accession.

The disease incidence was evaluated calculating the percentage index of diffusion (I%D).

3.3 EXPERIMENTAL INOCULATIONS

The experimental inoculations to test the level of resistance were assayed on plants cultivated in screenhouse, described earlier.

The parental plants of Mgaloblishvili and its progenies, 148 *V. vinifera* subsp. *vinifera* and 35 *V. vinifera* subsp. *sylvestris* were inoculated with populations of *P. viticola*. The reference varieties employed are Pinot noir, susceptible to *P. viticola,* and Bianca, as resistant control to assess their response to the pathogen.

3.3.1 Fungal material

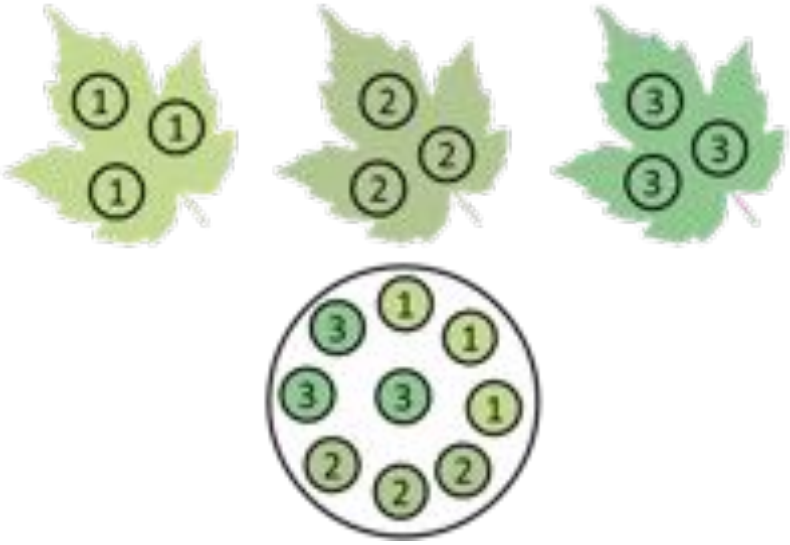
The *P. viticola* inoculum used in the experimental procedure was collected from naturally infected leaves of plants grown in vineyard plots not treated with fungicides against the downy mildew agent. The pathogen strains were collected from different vineyards located in Lombardy.

Symptomatic leaves were excised, placed in zip bags and transported to the laboratory in a ice box. The leaves were rinsed with running tap water to remove sporangia and incubated overnight in growth chamber at 22°C to induce fresh sporulation.

3.3.2 Experimental inoculations procedure

Experimental inoculations with *P. viticola* inoculum were carried out on leaf samples collected from the screenhouse at the beginning of grapevine growing seasons 2014, 2015 and 2016. Three leaves (3^{rd}-5^{th} leaf starting from the shoot apex) were detached from each accessions. Three leaf discs (15 mm diameter) were cut from each leaf with a cork borer and placed lower surface upward on a moistened filter paper placed in a Petri dish (6 cm diameter). Three plates containing three leaf discs were obtained for each grapevine genotype (Figure 3.4).

Figure 3.4: Scheme of leaf discs cutting and placement in the Petri dish



The leaf discs were sprayed with 1 mL *P. viticola* sporangia suspension ($5x10^4$ sporangia/mL) and incubated in growth chamber at 22 °C for 7- 10 days.

3.3.3 Data analysis

Each leaf disc was scored for the surface covered by sporulation at the stereo microscope (Leica Wild M10) by attributing the following classes: 0 = absence of sporulation; 1 = 0.1-2.5% of the surface covered by sporulation; 2 = 2.5-5%; 3 = 5–10%; 4 = 10–25%; 5 = 25–50%; 6 = 50–75%; and 7 = 75–100% of the leaf area covered by sporulation (Toffolatti *et al*., 2012).

The disease severity was estimated by the Percentage Index of Infections (I%I) calculated from the formula of Townsend and Heuberger (1947)

$$I\%I = \frac{\sum(n \cdot v)}{(n°class - 1) \cdot N} \cdot 100$$

where *n* is the number of leaf discs in each class, *v* the numerical value of each class and *N* represents the total number of leaf discs in the sample. The plants with I%I lower than 25% were considered resistant.

3.4 HISTOLOGICAL ANALYSES

Young detached leaves of cv Mgaloblishvili were placed on 1% water agar in Petri dishes, inoculated with numerous 10 µL droplets of sporangial suspension and incubated as previously described. The leaf areas under the droplets were collected from the inoculated samples with a cork borer (Ø 0.5 cm), at 4°C and stained with 0,05% aniline blue in 0.067 M K_2HPO_4 (pH 9) for 24 hours to investigate the callose deposition by the plant and the pathogen structures at 1, 2, 3 and 6 dai. Pinot noir, Bianca and Mgaloblishvili were kept at the same conditions and the collection of

the samples has been carried out at the same time with a standardized procedure, to evaluate the effective response of the plants and the development of the pathogen, avoiding that other factors modulating the results.

The samples were mounted in 75% glycerol on glass slides and observed under under UV light (Nikon Eclipse 80i equipped with a video-confocal system; Nikon Instruments S.p.a., Calenzano, FI, Italy) with DAPI filter, after staining for callose. The presence of autofluorescence was evaluated on unstained samples. The pathogen structures were visualized in blue, using DAPI filter, and the leaf tissues in red, using a FITC filter (ex 465–495 nm, dm 505 nm, ba 515–555). The same procedure was carried out on 'Bianca' and 'Pinot nero' as negative and positive controls.

3.5 ASSESSMENT OF THE AGGRESSIVENESS LEVEL OF PATHOGEN

Experimental inoculations on the susceptible and resistant reference varieties 'Pinot noir' and 'Bianca', and on Mgaloblishvili have been carried out with different *P. viticola* strains in a single day, to avoid the influence of environmental conditions on the output.

Eight *P. viticola* populations were collected from vineyards located in two different vineyards located in Santa Maria della Versa (PV), Belfiore (VR), Canevino (PV), Casarsa della Delizia (PN),Piateda (Fiorenza) (SO), Sondrio, Soave (VR) (Tab. 3.5).

Table 3.5: List of strains investigated on aggressiveness level

Strain	**Town**	**Province**
A	Santa Maria della Versa (Vineyard 1)	Pavia
B	Santa Maria della Versa (Vineyard 2)	Pavia
C	Belfiore	Verona
D	Canevino	Pavia
E	Casarsa della Delizia	Pordenone
F	Piateda	Sondrio
G	Sondrio	Sondrio
H	Soave	Verona

P. viticola strains were weekly propagated on detached leaves of cv 'Pinot noir' plants grown in screen house.

Seven leaves were collected respectively from 'Pinot noir' and 'Bianca' and from each leaf six leaf discs were obtained. Leaf discs belonging to one leaf were placed in six different Petri dishes, therefore each Petri dish is constituted by six leaf discsderived from six different leaves.

The aggressiveness level of these strains were estimated from the I%I calculated after visual assessment of the sporulating areas following the same protocol already described for the experimental inoculations.

The inoculum was considered aggressive, when showing I%I higher than 60% on Pinot noir. A medium level of aggressiveness was established when the I%I was included between 60 and 30%, a low level if I%I was lower than 30%.

3.5.1 Quantification of sporangia

The number of sporangia differentiated by the pathogen on the leaf discs of each grapevine cv, was calculated at 9 dai.

Sporangia were detached from the sporangiophores by vortexing each leaf disc in a 1.5 mL tube containing 500 µL of distilled water. The average number of sporangia (SN) per leaf disc was calculated from the average number of sporangia per mL of suspension determined by counting the spores in three replicates of 10 µL of sporangial suspension in a Neubauer counting chamber (Riechert Bright-Line haemocytometer, Hausser Scientific, Horsham, PA, USA) under a optical bright field microscope (Leitz Orthoplan).

3.6 ASSESSMENT OF P. viticola GENETIC DIVERSITY IN ITALY

During the grapevine growing season 2016, leaves showing downy mildew symptoms were randomly collected from 96 vineyards located in 13 different geographic regions in Italy.

In laboratory, diseased areas were excised with a 1 cm diameter cork borer and placed in 1,5 mL sterile Eppendorf tube. Each sample consisted of 1 leaf disc taken in correspondence of a single oil spot collected by a single leaf. The samples were stored at -20°C before lyophilization and then kept at room temperature until DNA extraction. The list of the *P. viticola* populations analyzed is given in Table.3.6.

Table 3.6: List of *P. viticola* populations analyzed with some characteristics: town, province, region, date of collection and varieties.

Town	**Province**	**Region**	**Varieties**	**Code number**
Adro	Brescia	Lombardy	Pinot nero	41
Adro	Brescia	Lombardy	Chardonnay	40
Adro	Brescia	Lombardy	Chardonnay (V.56)	22
Aldegheri-San'Ambrogio di Valpolicella	Verona	Veneto	Garganega	70
Aldegheri-San'Ambrogio di Valpolicella	Verona	Veneto	Chardonnay	67
Strain C	Verona	Veneto	unknown	35
Borgonato di corte franca	Brescia	Lombardy	Pinot nero (C4)	5
Borgonato di corte franca	Brescia	Lombardy	Chardonnay	91
Camignone di Passirano	Brescia	Lombardy	Chardonnay	68
Canale	Cuneo	Piemont	Dolcetto	62

Town	Province	Region	Varieties	Code number
Canale	Cuneo	Piedmont	Bonarda	74
Strain D	Pavia	Lombardy	Barbera	53
Cappella di Sant'Andrea (Palagio)- San Gimignano	Siena	Tuscany	Sangiovese	43
Cappella di Sant'Andrea- San Gimignano	Siena	Tuscany	Cabernet	80
Cappella di Sant'Andrea- San Gimignano	Siena	Tuscany	Casale-Sangiovese	55
Strain E	Pordenone	Friuli-Venezia Giulia	Pinot noir	8
Casarsa della Delizia tesi B	Pordenone	Friuli-Venezia Giulia	Pinot noir	10
Castelnuovo Berardenga	Siena	Tuscany	Sangiovese I	58
Castelnuovo Berardenga	Siena	Tuscany	Cigliegiolo	26
Castelnuovo Berardenga	Siena	Tuscany	Sangiovese II	50

Town	Province	Region	Varieties	Code number
Castelnuovo Berardenga	Siena	Tuscany	Sangiovese III	60
Castelvenere	Benevento	Campania	Aglianico	57
Castiglion del Bosco - Montalcino-S.Anna	Siena	Tuscany	unknown	27
Castiglion del Bosco - Montalcino 2	Siena	Tuscany	unknown	17
Castiglion del Bosco – Montalcino-Gauggiole	Siena	Tuscany	unknown	89
Castiglion Fiorentino	Arezzo	Tuscany	Sangiovese	81
Castiglion Fiorentino	Arezzo	Tuscany	Merlot	6
Castiglion Fiorentino	Arezzo	Tuscany	Chardonnay	39
Castiglion Fiorentino2	Arezzo	Tuscany	Chardonnay	46
Castiglion Fiorentino	Arezzo	Tuscany	Breeding Manzoni	47
Collazzone	Perugia	Umbria	Malvasia nera	16
Due Carrare 1	Padova	Veneto	Merlot	19

Town	Province	Region	Varieties	Code number
Due Carrare 2	Padova	Veneto	Merlot	52
Due Carrare	Padova	Veneto	unknown	21
Erbusco	Brescia	Lombardy	Chardonnay (SM73)	31
Erbusco - Cà del Bosco	Brescia	Lombardy	Chardonnay (C4)	7
Erbusco - Cà del Bosco	Brescia	Lombardy	Pinot nero	30
Erbusco - Cà del Bosco	Brescia	Lombardy	unknown	13
Erbusco - Cà del Bosco	Brescia	Lombardy	Chardonnay (CH3)	34
Fondazione Foianini	Sondrio	Lombardy	Nebbiolo	24
Gaiole in Chianti	Siena	Tuscany	Sangiovese	51
Gaiole in Chianti2	Siena	Tuscany	Sangiovese	56
Gaiole in Chianti	Siena	Tuscany	Sangiovese II	29
Gaiole in Chianti3	Siena	Tuscany	Sangiovese	75
Gaiole in Chianti-Bicocchi (Piazzine)	Siena	Tuscany	Sangiovese	54
Gambellara (La Biancara)	Vicenza	Veneto	Garganega	20
Gattico	Novara	Piedmont	Nebbiolo	32
Grisì (Monreale)	Palermo	Sicily	Cataratto	76
Grisì (Monreale)	Palermo	Sicily	Merlot	69
Town	**Province**	**Region**	**Varieties**	**Code number**

Milano (UNIMI-orto)	Milano	Lombardy	unknown	9
Mombaruzzo 1	Asti	Piedmont	Moscato	14
Mombaruzzo 2 (Roero)	Asti	Piedmont	Moscato	25
Mombaruzzo 3	Asti	Piedmont	Moscato	37
Montefalco	Perugia	Umbria	Sagrantino	84
Montefalco	Perugia	Umbria	Grechetto	83
Montefalco	Perugia	Umbria	Sangiovese	79
Montenidoli (Fidanza)-San Gimignano	Siena	Tuscany	Sangiovese	73
Montenidoli (Fidanza)- San Gimignano2	Siena	Tuscany	Sangiovese	86
Montenidoli (Riviera)- San Gimignano	Siena	Tuscany	Vernaccia	88
Montenidoli (Riviera)- San Gimignano	Siena	Tuscany	Sangiovese	15
Monzambano	Mantova	Lombardy	Merlot	66
Monzambano - San Pietro	Mantova	Lombardy	Chardonnay	92

Town	Province	Region	Varieties	Code number
Murisengo	Alessandria	Piedmont	Barbera	64
Murisengo2	Alessandria	Piedmont	Barbera	72
Napoli-Campi Flegrei- collina Camaldoli	Napoli	Campania	Falanghina	12
Ortovero- vigneto Annunziata	Savona	Liguria	Pigato	4
Ortovero- vigneto Garaxin	Savona	Liguria	Pigato	78
Panzano in Chianti (Greve)-Candialle (sopra cipresso)	Firenze	Tuscany	Sangiovese	49
Strain F	Sondrio	Lombardy	unknown	36
Pieve San Nicolò-Ponti sul Mincio	Mantova	Lombardy	Pinot grigio	85
Piglio	Frosinone	Lazio	unknown	48
Pozzolengo (Cà dei Frati)	Brescia	Lombardy	Lugana	38
Pozzolengo (Marangona)	Brescia	Lombardy	Lugana	33
Provaglio d'Iseo	Brescia	Lombardy	Chardonnay	63
Radda in Chianti	Siena	Tuscany	Sangiovese	11
Radda in Chianti	Siena	Tuscany	Trebbiano	1

Town	**Province**	**Region**	**Varieties**	**Code number**
Radda in Chianti-Montevertine	Siena	Tuscany	Sangiovese	23
Retorbido	Pavia	Lombardy	unknown	2
Santa Giuletta-Travaglina	Pavia	Lombardy	Croatina	90
Strain A	Pavia	Lombardy	unknown	42
Sassari A3	Sassari	Sardinia	unknown	93
Sassari B1	Sassari	Sardinia	unknown	94
Sassari F7	Sassari	Sardinia	unknown	95
Sassari G4	Sassari	Sardinia	unknown	96
Serralunga d'Alba	Cuneo	Piedmont	Chardonnay	65
Serralunga d'Alba	Cuneo	Piedmont	Pinot nero	71
Strain H	Verona	Veneto	unknown	77
Valenzano-Passirano	Brescia	Lombardy	Chardonnay	3
Finale Ligure	Savona	Liguria	unknown	61
Vorno-Tenuta dello Scompiglio	Lucca	Tuscany	Sangiovese I	44
Vorno-Tenuta dello Scompiglio	Lucca	Tuscany	Sangiovese II	82
Vorno-Tenuta dello Scompiglio	Lucca	Tuscany	Sangiovese III	18

Town	Province	Region	Varieties	Code number
Vorno-Tenuta dello Scompiglio	Lucca	Tuscany	Sangiovese IV	87
Vorno-Tenuta dello Scompiglio	Lucca	Tuscany	Canaiolo Colorino	28
Vorno-Tenuta dello Scompiglio	Lucca	Tuscany	Syrah	45
unknown	unknown	Abruzzo	unknown	59

3.6.1 DNA extraction

At the "Institution National de la Recherche Agronomique" (INRA) – Bordeaux – Aquitaine, the total DNA was extracted from single lyophilized oil spot for each sample using the protocol developed by Delmotte *et al.* (2006).

Two sterile glass beads were added to each tube. The leaf discs were then pulverized with a mixer mill for 1 minute at 25 cps. The samples were briefly centrifuged at 3700 rpm before adding 400 µl of CTAB buffer.

CTAB buffer final concentration:

Tris pH8	1M pH8
EDTA (Ethylenediaminetetraacetic acid) pH8	0.5 M pH8
CTAB (cetyltrimethyl ammonium bromide)	2 %
PVP-40 (polivinilpyrrolidone)	4 %
NaCl	1,5 M

The suspension was incubated at 65°C for one hour and an half, amended with 400 µl chloroform:isoamyl alcohol 24:1, mixed for one minute and then centrifuged for 30 minutes at 3700 rpm at 4°C.

The supernatant was recovered in a 2 ml sterile Eppendorf tube and the suspension was added with 2/3 volume (200µl) of cold Isopropanol. The tubes were mixed by inversion for 1 minute and centrifuged at 2300 rpm for 30 minutes at 4°C. After discarding the supernatant, the precipitate was rinsed with 200 µl of 70% cold ethanol, by gently pipetting. The suspension was centrifuged for 10 minutes at maximum speed at 4°C and then the supernatant was discorded.

The tubes were placed in a SpeedVac (Thermo Scientific) for 5 minutes in order to dry the DNA pellet. The DNA extracted was re-suspended in 50 µl of sterile pure water and conserved at 4°C.

3.6.2 Microsatellite amplification

The 106 *P.* viticola populations were genotyped for 32 microsatellite loci developed by Gobbin *et al.,* (2003), Delmotte *et al.,* (2006) and Rouxel *et al.,* (2012). Thus, 32 different primer pairs were used to analyze the genetic differences of *P. viticola* populations. The PCR reactions were carried out with forward primers conjugated with fluorescent dyes (Table 3.7).

Table 3.7: Main characteristics of the 32 microsatellite loci used in this study: locus name, GeneBank Accession no., primer sequence, core repeat, fluorescent dye used, annealing temperature (T_a), size range of alleles (bp).

Locus	GeneBank Accession N°	Primer sequence (5'-3')	Repeat motif	Marquage	T_a (°C)	Size range alleles (bp)
Pv14 ★	DQ217577	F:CAGAAACGCACAAGGTCTGA R:AATTGCATACTGCAGCAACG	(TG)6	VIC	54°C	120-126
ISA ●		F:ATTAGCGGCATGGACGTT R:GAGAAGTTCCGCCAAGTACA	(TC)n	PET	54°C	112-138
Pv17 ★	DQ217579	F:CAGAGTCGAACAAGTACATT R:CTTTGTCGCCTTCTAACAAC	(TC)12	6FAM	54°C	140-150
Pv39 ★	DQ217581	F:ACGCATGGCGAACACGTAAG R:CAGACGGGAAGAAGTTGCTC	(CA)6	VIC	54°C	163-185
Pv31 ★		F:CCCCATGCTGAAGAGTTTC R:TTCTTTCTAAGGCCGTGTGG	(CA)9	6FAM	54°C	236-240
Pv16 ★	DQ217578	F:TAAAAATATGGTGGCGTCAG R:CAGCAGTCTCCGTCTCATCAG	(TGCTGTTGC)2(TGC)2	PET	54°C	238-249
Pv7 ★	DQ217575	F:TCTTCCGAAAAGGGACGTAA R:GCGTCACTGCATCTACGAAA	(TG)5	6FAM	54°C	281-307
Pv65 ▲	JQ219972	F:CTTTGGCCCACGTCATAGTT R:CGCTTTCGGTAGGTCCATTA	(TC)9	NED	57°C	196-202
Pv67 ▲	JQ219973	F:GCATTGAGCAGACACCTTGA R:GAGCGATAAGACCACAAATAGTGA	(AC)9	6FAM	54°C	348-368
Pv74 ▲	JQ219984	F:GCAACGTTGTGCAAGCTTTA R:GCATTATGATGGAGCTCACG	(AG)7	6FAM	54°C	176-182
Pv76 ▲	JQ219974	F:CTGGTTGCTGATGCACTGAC R:GGCGGTGACTAAGTCGTTGT	(TC)7	VIC	57°C	136-140
Pv83 ▲	JQ219985	F:TGCAGCATTGTTTCATCCAT R:ACACGGTACTTTGCGTTCCT	(TG)6	VIC	54°C	238-242
Pv87 ▲	JQ219986	F:CGTGCAATTCAAACAACAGG R:CTCACAAGGACGACTGGACA	(CT)6	NED	54°C	152-154
Pv88 ▲	JQ219987	F:AATACCAAAAATGGCCGTCA R:ACTCTCTTGCCAGCACCATC	(GT)6	6FAM	54°C	202-208
Pv91 ▲	JQ219975	F:ACCAGCCTTTGCGAAGATAA R:TGAAAGTTACGTGTCGCACC	(TG)6	6FAM	54°C	142-146
Pv93 ▲	JQ219976	F:TAGCACCGGACTAGGCGTAT R:TGTACCCTGTTGCCCTCTTC	(GT)6	6FAM	54°C	147-151
Pv101▲	JQ219979	F:AACACGGCGCCAAAGTATTA R:GGGCATTAACGTGCAAATTC	(CTT)6	VIC	54°C	263-266

Pv103▲	JQ219981	F:TGACCTACCACCCATTTACCA R:ACGGTCAGGTCAAAAGCAGT	(TG)6	PET	54°C	277-299
Pv104▲	JQ219982	F:CTACGCTCGAGGATGACACA R:GACATTGCCGCACCTAAGAT	(CA)6	VIC	54°C	321-324
Pv126▲	JQ219989	F:GCTCTCTGCAGGACGTTTTT R:GCCGTTCTTCACGTTCTAGC	(GAC)10	PET	50°C	182-206
Pv127▲	JQ219990	F:TTGAAAACGCGGATAGGAAC R:GAACGTCCAGTTCGGATTGT	(CA)9	VIC	54°C	213-223
Pv134▲	JQ219992	F:CATGCTCACGTAGACCTCCA R:AATGCAGAGCTCCCATAACG	(AG)6	6FAM	54°C	220-226
Pv137▲	JQ219995	F:AAGTGGGACACATCAAGCGT R:TGGCAATAAGTTTATGCCTCG	(AT)9	NED	57°C	243-256
Pv138▲	JQ219996	F:CGTGGATCATGACGTTTGTC R:CGACGAATCAGGGACAAGAT	(TA)9	6FAM	57°C	225-235
Pv139▲	JQ219997	F:GACCCGGACAATGGACTCTA R:CCGCCATGTATTGAACAGTG	(AC)8	6FAM	57°C	126-133
Pv140▲	JQ219998	F:GCTTGAGAAGAATGGAACGC R:CCCAGAAGGGTGATACGAGA	(TA)9	VIC	57°C	172-201
Pv141▲	JQ219999	F:ACGACGACATGAGCTGTACG R:GAAGGTGGTGTCATGGGTTT	(TC)9	VIC	57°C	190-192
Pv142▲	JQ220000	F:TTATGCCACGCAAATCTCTG R:AGGGCGAAATACGAGAGTGA	(CT)11	NED	57°C	209-219
Pv143▲	JQ220001	F:CCTGAATAAAGCAACACGCA R:TTGGCAGCAAATTGTACGAC	(AT)8	6FAM	57°C	121-135
Pv147▲	JQ220005	F:TCGACTACGAGTCCGAGAGG R:TTCTAGCTCGACGAAGACCG	(TCGACT)8	NED	57°C	189-219
Pv148▲	JQ220006	F:CGACCTATGTTTCGCCATTT R:GAGTCGTCGTAGAAGGCGTC	(ACA)6	PET	57°C	134-137

★ Delmotte *et al.,* 2006

● Gobbin *et al.,* 2003

▲ Rouxel *et al.,* 2012

The primers were multiplexed in 5 different “primer mix” tunes as described in Table 3.8.

Table 3.8: The list of 5 primer mix

MIX 1 LOCI	DYE
PV14	green
ISA	red
PV17	blue
PV39	green
PV31	blue
PV16	red
PV7	blue

MIX 2 LOCUS	DYE
PV138	blue
PV140	green
PV143	blue
PV147	black
PV101	green
PV103	red
PV74	blue

MIX 3 LOCUS	DYE
PV135	blue
PV137	black
PV141	green
PV93	blue
PV65	black
PV148	red
PV104	green

MIX 4 LOCUS	DYE
PV139	blue
PV76	green
PV87	black
PV126	red
PV88	blue
PV83	green

MIX 5 LOCUS	DYE
PV127	green
PV134	blue
PV67	blue
PV142	black

PCR amplifications were carried out in a final 6 μl reaction volume, with a primer concentration of 10mM, as follows:

ddH_2O	2.5 μl
2 X Multiplex PCR MASTER MIX (Qiagen)	1.5 μl
Primer mix (10 mM)	0.5 μl
DNA	1.5 μl

PCR was performed in an Eppendorf Mastercycler ep (Eppendorf, Germany) using the following conditions: an initial cycle of denaturation at 95°C for 15 minutes, followed by 35 cycles of 30 seconds at 94°C, 1 minute at 55°C (annealing) and 72°C (extension) for 45 seconds. These step is followed by a final elongation at 60°C for 30 minutes.

The PCR products were diluted adding 50 μl of ddH_2O. Positive (the DNA of three *P. viticola* strains) and negative (ddH_2O) controls were included in each experiment.

1.5 μl of diluted PCR products were transfered in a specific ABI plate for fragment size analysis with 10 μl of HI-DI Formamide (Life Technologies) and 0.2 μl of Genescan-600 LIZsize standard (Life Technologies).

Fragment size analysis was performed in an automated capillary genetic analyzer-sequencer 3130 (Applied Biosystems).

The main sizes of the alleles per lead locus are shown in Table 3.9.

Table 3.9: Size of alleles found in literature (in bold the most frequent)

Locus	Size of alleles
ISA	112, 114,118, **120,122,124,** 126, 130, 132, 134, 136, 138
Pv14	**120, 122, 124**
Pv16	238, 240, **246, 249**
Pv17	140, **142, 144, 146,** 148, 150
Pv31	236, **238, 240**
Pv39	163, 169, **175, 177,** 183, 185
Pv7	284, **286, 288,** 307
Pv101	**263, 266**
Pv103	**288, 298**
Pv74	127, 129, 131, 133, 135
Pv147	195, 201, 209, 210, 213, 219
Pv138	229, 231, 233
Pv140	188, 192, 194, 196, 198
Pv143	127, 129, 131, 133, 135, 137
Pv104	322
Pv65	194, **196**, 202
Pv39	**146, 150,** 152
Pv148	126, 134, 137

Pv135	217, 220
Pv137	246, 248, 250, 252, 254
Pv141	190, 192
Pv126	194, 200
Pv76	135, **139**
Pv83	238, 240, 242
Pv87	152, 154
Pv88	204, 206
Pv139	129, 131, 133
Pv127	215, 217, 219, 223
Pv134	222, 224
Pv142	209, 211, 215, 219
Pv67	348, 366

3.7 DATA ANALYSIS

The normal distribution and homogeneity of variances of means of quantitative variables were verified using respectively the Shapiro-Wilk test and the Levene's test by using SPSS V.23.

The distribution of I%I values obtained by natural infections in field evaluation and by experimental inoculation assay were visualized by box plot graphs.

Differences between the average values of I%I obtained in the experimental inoculations carried out for testing the aggressiveness level of the strains, were analysed by non parametric analysis of variance by ranks, the Kruskal-Wallis test, because the normal distribution of these data could not be assumed. The average of sporangia/cm^2 produced by the pathogen on the three accessions were compared using one-way ANOVA

and multiple comparison of the means with REGW-F test (Rayan-Einot-Gabriel-Welsh F).

In all cases, the differences were considered significant with α=0.05.

3.7.1 Population structure analysis

Nei's genetic identity (*I*) (Nei, 1972) and genetic differentiation (F_{ST} measured via analysis of molecular variance) (Peakall *et al.,* 1995) were determined among *P. viticola* samples using GenAlEx v.6.501 on full data sets. Principal coordinates analysis (PCoA) was carried out using GenAlEx v. 6.501 on pairwise genetic distances between all genotypes in order to visualize the major patterns of variation within and among populations. Nei genetic identity is the normalized identity of genes between two populations and varies between 0 (the compared populations are different), and 1 (the compared populations are identical).

The existence of a population structure in the total sample was further investigated using the Bayesian approach implemented in Structure Version 2.3.4 (Pritchard *et al.,* 2000; Falush *et al.,* 2003). This clustering algorithm assumes a model in which there are populations (where *K* may be unknown), each of which is characterized by a set of allele frequencies at each locus. Individuals in the sample are assigned probabilistically to these *K* populations, or jointly to two or more populations if their genotypes indicate that they are admixed, without consideration of their region of sampling. *K* varied from 1 to 8, each with 10 independent simulations to check the consistency of the results. Each simulation consisted in 1 000 000 Monte-Carlo Markov Chain (MCMC) iterations preceded by a burn-in period of 500 000 iterations. According to Evanno *et al.* (2005) the best estimation of *K* was that associated with highest ΔK,

an ad hoc quantity related to the second order rate of change of the log probability (likelihood) of the data and was calculated by Structure Harvester (Earl and vonHoldt, 2012).

On the genotyped *P. viticola* strains, dendrograms were drawn using Mega4 (Tamura *et al.,* 2007) under the clustering rule of the Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm.

The software package Coancestry (Wang, 2011) implements seven relatedness estimators and three inbreeding estimators to estimate relatedness and inbreeding coefficients from multilocus genotype data. The program to simulate multilocus genotype data of individuals with a predefined relationship, and to compare the estimators and the simulated relatedness values to facilitate the selection of the best estimator in a particular situation. Bootstrapping and permutations are used to obtain the 95% confidence intervals of each relatedness or inbreeding estimate (Wang, 2011).

4. RESULTS

4.1 NATURAL INFECTIONS IN FIELD EVALUATION

The behavior of 94 Georgian varieties cultivated in open field at the Research Centre of Riccagioia, in Torrazza Coste (PV) was evaluated as a consequence of natural infections.

Figure 4.1 Box plot distribution of the I%D values recorded in field on leaves The I%D of the 'Croatina N' plot (CRO) are also indicated.

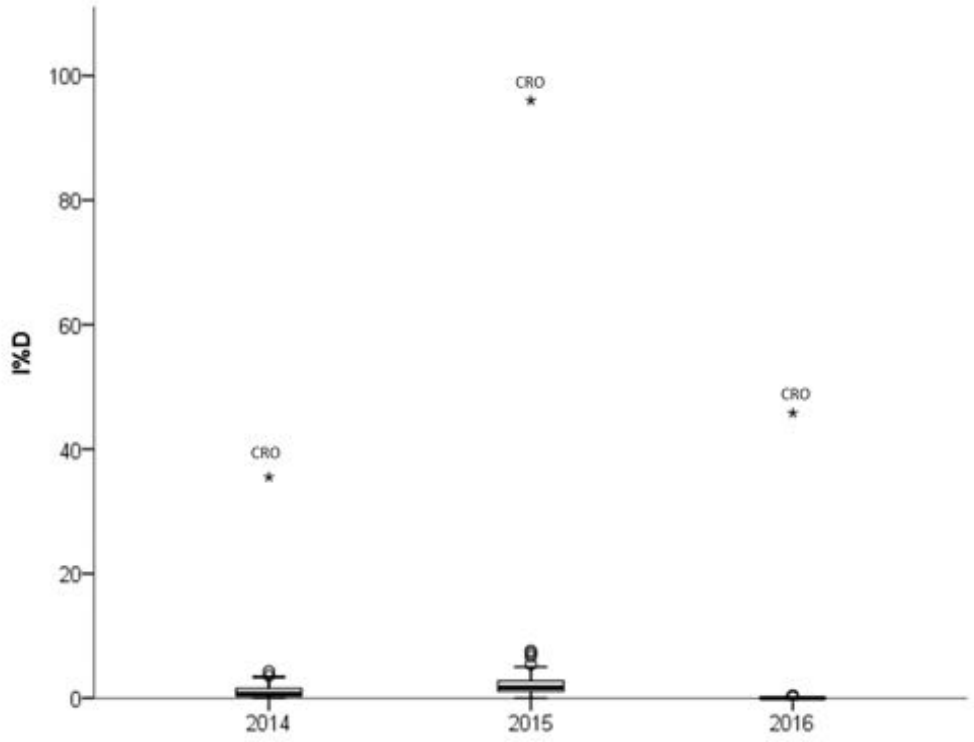
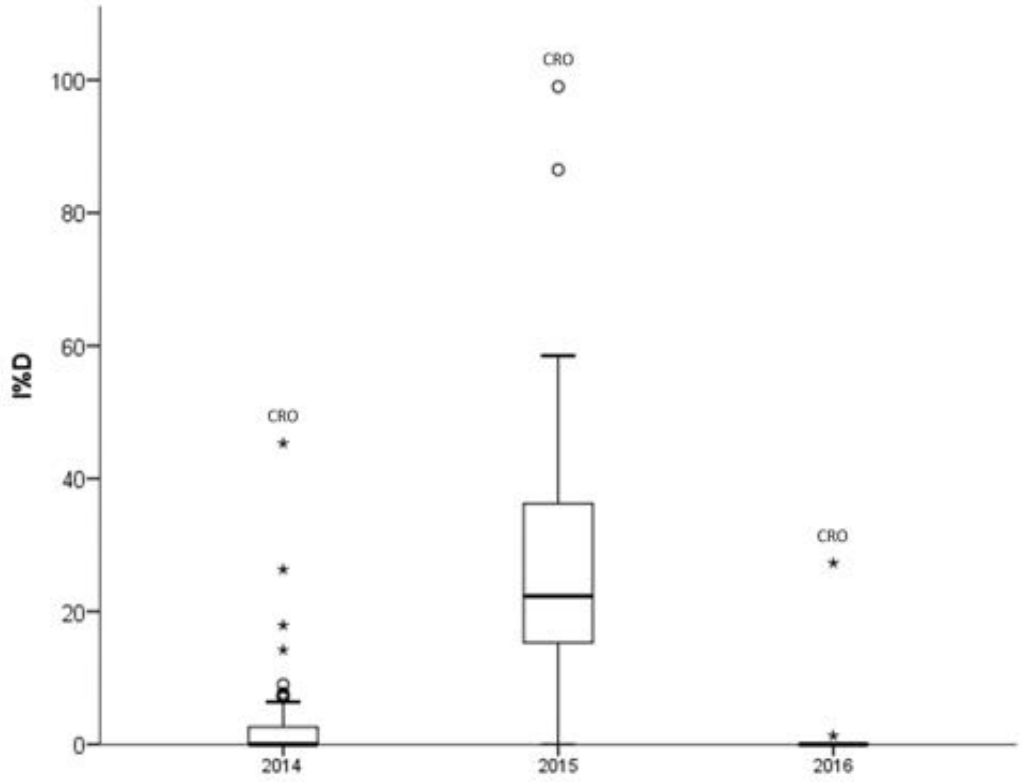


Figure 4.2 Box plot distribution of the I%D values recorded in field on bunches The I%D of the 'Croatina N' plot (CRO) are also indicated.



In Figure 4.1 and 4.2 are represented the distributions of the accessions in relation with their I%D on leaves and bunches estimated during the vegetative seasons of 2014, 2015 and 2016.

The occurrence of natural infections on Georgian accessions was very low on leaves in 2014, 2015 and 2016. The I%D were lower than 8 %.

In the 'Croatina N' plot, used as susceptible control, 35% of the leaves was affected by the disease in 2014. The I%D on leaves were higher than 95% in 2015 and than 45% in 2016 (Fig. 4.1). The values showed by Georgian accessions severely diverged by those observed on 'Croatina N'. Even in 2015, when the disease pressure was particularly high, as shown by the susceptible control.

Vitis labrusca L. (M24D), the resistant control variety, did not show any downy mildew symptoms during whole period of investigation.

Mgaloblishvili (L22A) confirmed the resistant behavior to natural infection already observed in preliminary assays showing I%D values ranging from 0.2% (2016) to 1.7% (2014). (Toffolatti *et al*., 2016).

The downy mildew incidence on bunches was particularly low in 2014 and 2016 (Fig. 4.2). In the 'Croatina N' plot the I%D on bunches were higher than 45% in 2014. More severe downy mildew epidemics occurred in 2015, when the 99% of affected bunches were observed in 'Croatina N'. On the contrary, the I%D on bunches in 2016 were lower than 28%.

Also on bunches the pathogen diffusion was reduced, with some exceptions. No cultivars showed I%D close or higher than those observed in 'Croatina N' in whole years. Georgian varieties showed I%D on bunches lower than 26.5% in 2014. In 2015, when the disease pressure was particularly high, 50% of the Georgian accessions showed a reduced percentage of infected bunches with I%D lower than 22%. The other accessions showed I%D ranging from 23 to 86%. Particularly interesting are 23 accessions with I%D lower than 14% among which there is Mgaloblishvili.

In 2016 no bunches showed disease symptoms except for a cultivar (L23A) which recorded I%D equal to 1,3%.
Vitis labrusca showed not bunch infection, and Mgaloblishvili showed a low number of infected bunches in 2015 and not infection at all in 2014 and 2016.

4.2 SCREENING OF ACCESSIONS FOR RESISTANCE TO P. viticola

Grapevine varieties (*V. vinifera* L.) coming from Caucasus and Iran were selected as representative of east Europe grapevine germplasm and were cultivated in DiSAA greenhouse. The screening analysis were carried out during 2014, 2015 and 2016.

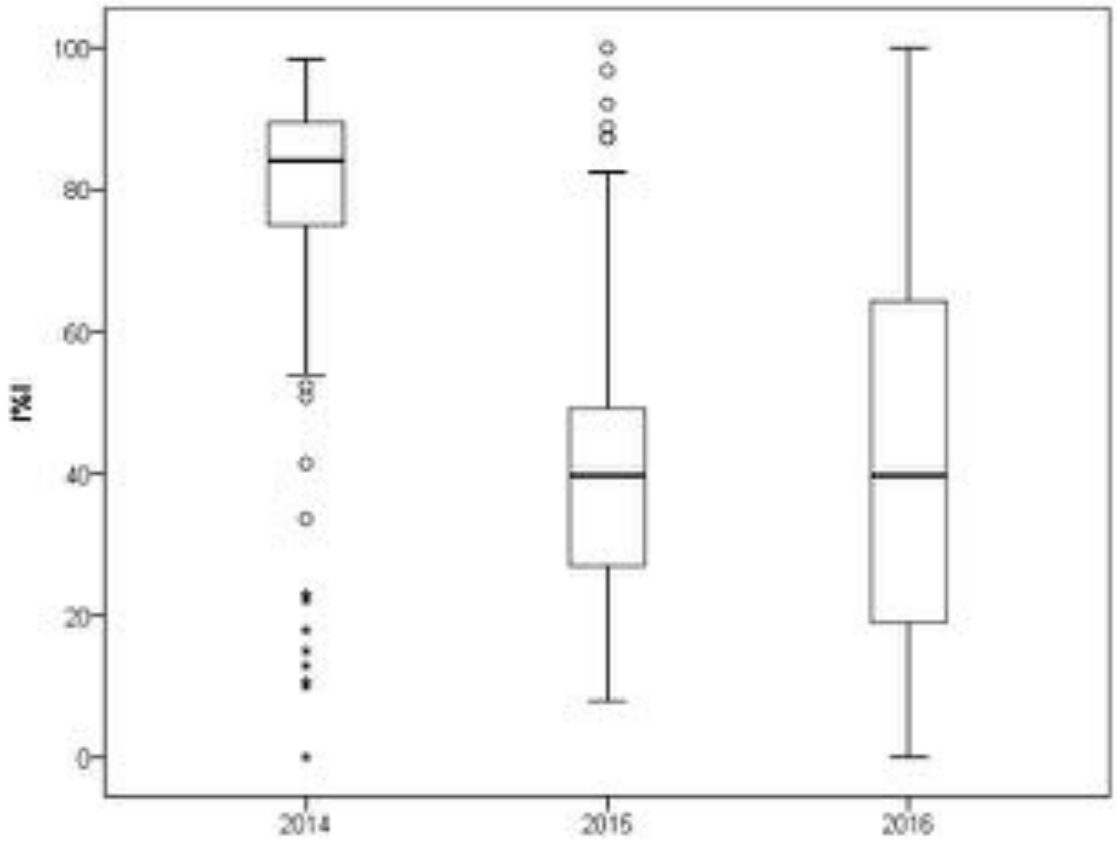
The number of accessions tested was 199 in 2014, 176 in 2015, 177 in 2016.

The screening for resistance was carried out on cultivated and wild Caucasian and some Iranian accessions.

Based on the results obtained by the visual assessment of disease severity, the Percentage Index of Infection (I%I) was calculated for each accession. The distribution of the data related to the I%I, obtained by the experimental inoculations on 9 leaf discs per accession, are visualized by box plots.

4.2.1 Caucasian and Iranian V. vinifera subsp. vinifera

Figure 4.3 Box plot distribution of the I%I of Caucasian and Iranian cultivated accessions from 2014 until 2016



In Figure 4.3 are represented the distribution of the I%I of the accessions. Most of the accessions showed high levels of susceptibility to the pathogen (I%I >25%). The average I%I values estimated showed a wide range of distribution, from 0 to 100%.

During the first year the I%I values obtained were very high apart from a few outliers, the I%I of the accessions ranged from 57 to 99%: only a fiew (outlier) accessions showed a resistant behaviour, with I%I lower than 25% (Table 4.1).

Table 4.1: I%I values of the accessions which resulted resistant in 2014.

CULTIVAR	COUNTRY	ACCESSION CODE	I%I
Dondghlabi Shavi	Georgia	**2-17 A**	0.0
Institutis Grdzelmtevana	Georgia	**37-1**	10.0
Almura Tetri	Georgia	**3-10 B**	10.7
Kharistvala Meskhuri	Georgia	**1-37 B**	12.9
Dzvelshavi	Georgia	**2-13 A**	15.0
Rkatsiteli Tsiteli	Georgia	**1-17 A**	17.9
Saperavi Khashmis	Georgia	**SK**	22.1
Gorula (clone)	Georgia	**1-38 A**	22.9
Borchalo	Georgia	**32-2**	22.9

During 2015 the accessions showed lower levels of susceptibility. 19% of the accessions tested showed a resistant behaviour and are listed in Table 4.3.

Table 4.2 I%I values of the accessions which resulted resistant in 2015

CULTIVAR	COUNTRY	ACCESSION CODE	I%I
Portoka	Georgia	**F05C08**	7.9
Shavkapito	Georgia	**F06C06**	9.5
Djvari	Georgia	**F02C03**	11.1
Saperavi	Georgia	**F05C11**	14.3
Chesmgave	Iran	**F27C05**	14.3
Endeladziseuli	Georgia	**F11C16**	15.9
Chicvi	Georgia	**F15C09**	15.9
Abak	Iran	**F27C03**	15.9
Vazisubnis tsiteli	Georgia	**F05C07**	17.5
Shavi qurdzeni	Georgia	**F06C17**	17.5
Mamukasvazi	Georgia	**F07C17**	17.5
Tchodi salkhinosi	Georgia	**F08C11**	17.5
Akomshtali	Georgia	**F08C22**	17.5
Skhilatubani	Georgia	**F09C05**	17.5
Tsitska	Georgia	**F02C06**	19.0
Almura tetri	Georgia	**F03C11**	19.0
Aladasturi	Georgia	**F06C04**	19.0
Kornistvala	Georgia	**F11C02**	19.0
Tsolikouri mtsvivani	Georgia	**F02C19**	20.6

CULTIVAR	COUNTRY	ACCESSION CODE	I%I
Argvetuli sapere	Georgia	**F05C04**	20.6
Badagi	Georgia	**F07C06**	20.6
Khupishij	Georgia	**F08C21**	20.6
Kumsi tetri	Georgia	**F15C19**	22.2
Tsirkvalis tetri	Georgia	**F01C10**	23.8
Bzvanura	Georgia	**F03C07**	23.8
Odjaleshi	Georgia	**F09C06**	23.8
Alexandrouli	Georgia	**F12C19**	23.8
B603	Iran	**F26C04**	23.8

None of the resistant of 2014 confirmed the same behaviour in 2015. During the last year activity the distribution of the data was more ample: the accessions that showed resistant behaviour are listed in the table 4.5.

Table 4.3 I%I values of the accessions which resulted resistant in 2016

CULTIVAR	COUNTRY	ACCESSION CODE	I%I
Gorula	Georgia	**F01C17**	0.0
Kochtura	Georgia	**F15C10**	0.0
Chitistvalakakhuri (bobduri)	Georgia	**F16C10**	0.0
Andreulitkhelkana	Georgia	**F16C18**	0.0

CULTIVAR	COUNTRY	ACCESSION CODE	I%I
Yaghotiwhite	Iran	**F27C08**	0.0
Kumsi tetri	Georgia	**F15C15**	1.6
Mkhargrdzeli	Georgia	**F16C08**	1.6
Mskhviltvala tetri	Georgia	**F16C15**	1.6
Chesmgave	Iran	**F27C05**	3.2
Tvaldamtsvriseuli	Georgia	**F04C21**	4.8
Orbeluriodjaleshi	Georgia	**F08C03**	4.8
Chitistvalakakhuri	Georgia	**F01C05**	6.3
Tsirkvalis tetri	Georgia	**F01C10**	6.3
Saperavi mskhvilmartsvala	Georgia	**F11C04**	6.3
Gldanula (gorula)	Georgia	**F16C09**	7.9
Mtsvanekakhuri	Georgia	**F01C18**	7.9
Khupishij	Georgia	**F08C21**	7.9
Chesmgave	Iran	**F26C03**	7.9
Grubela kakhuri	Georgia	**F04C20**	11.1
Adreulatchelkana	Georgia	**F16C21**	11.1
Dzvelshavi	Georgia	**2-13 A**	11.1
Almura tetri	Georgia	**F03C11**	11.1
Budescuri tetri	Georgia	**F15C08**	12.7
Ghrubelakartlis	Georgia	**F04C22**	12.7

CULTIVAR	COUNTRY	ACCESSION CODE	I%I
Abshiluri/avshiluri	Georgia	**F06C16**	12.7
Batomura	Georgia	**F08C12**	12.7
Bazaleturi	Georgia	**F02C20**	14.3
Tsitelouri	Georgia	**F08C10**	14.3
Alexandrouli	Georgia	**F12C19**	14.3
Sirgula	Georgia	**F02C14**	15.9
Borchalo	Georgia	**32-2**	15.8
Almura tetri	Georgia	**F15C11**	15.9
Matchkvaturitskhakaiasi	Georgia	**F11C07**	17.5
Mugiuretuli	Georgia	**F11C11**	17.5
Bua kurdzeni	Georgia	**F16C19**	17.5
Kumsi tetri	Georgia	**F15C19**	19.0
Almura Tetri	Georgia	**3-10B (G36)**	19.0
Gomis tetri	Georgia	**F01C16**	20.6
Chekardani	Georgia	**F15C13**	20.6
SaperaviKhashmis	Georgia	**SK**	22.2
Shavkapito	Georgia	**F06C06**	22.2
Saperavi	Georgia	**F05C11**	23.8
Akomshtali	Georgia	**F08C02**	23.8
Kashmissaperavi	Georgia	**F11C22**	23.8
Kapistoni tsitsiliani	Georgia	**F15C18**	23.8

None of the resistant accessions found in a single year showed the same behaviour during all the three years. However, some accessions that were resistant in 2014, showed the same behaviour in 2016 (Table 4.6).

Table 4.6 I%I values of the accessions which confirmed the resistance in 2014 and 2016

CULTIVAR	COUNTRY	ACCESSION CODE	I%I 2014	I%I 2016
Dzvelshavi	Georgia	**2-13 A**	15.0	11.1
ɔndghlabi Shavi	Georgia	**2-17 A**	0.0	30.1
Almura Tetri	Georgia	**3-10 B**	10.7	19
Borchalo	Georgia	**32-2**	22.9	15
Institutis Grdzelmtevana	Georgia	**37-1**	10.0	39
SaperaviKhashmis	Georgia	**SK**	22.1	22.2

Moreover some accessions that resulted resistant in 2015 showed low I%I values in 2016, confirming the resistant character (Table 4.7).

Table 4.7 I%I values of the accessions which confirmed the resistance in 2014 and 2016

CULTIVAR	COUNTRY	ACCESSION CODE	I%I 2015	I%I 2016
Tsirkvalis tetri	Georgia	**F01C10**	23.8	6.3
Tsolikouri mtsvivani	Georgia	**F02C19**	20.6	25.4
Almura tetri	Georgia	**F03C11**	19	11.1
Saperavi	Georgia	**F05C11**	14.3	23.8
Shavkapito	Georgia	**F06C06**	9.5	22.2
Opoura	Georgia	**F08C02**	25.4	23.8
Khupishij	Georgia	**F08C21**	20.6	7.9
Matchkva turitskhakaiasi	Georgia	**F11C07**	25.4	17.5
Alexandrouli	Georgia	**F12C19**	23.8	14.3
Kumsi tetri	Georgia	**F15C19**	22.2	19.0
Chesmgave	Iran	**F27C05**	14.3	3.2
Yaghotiwhite	Iran	**F27C08**	25.4	0.0

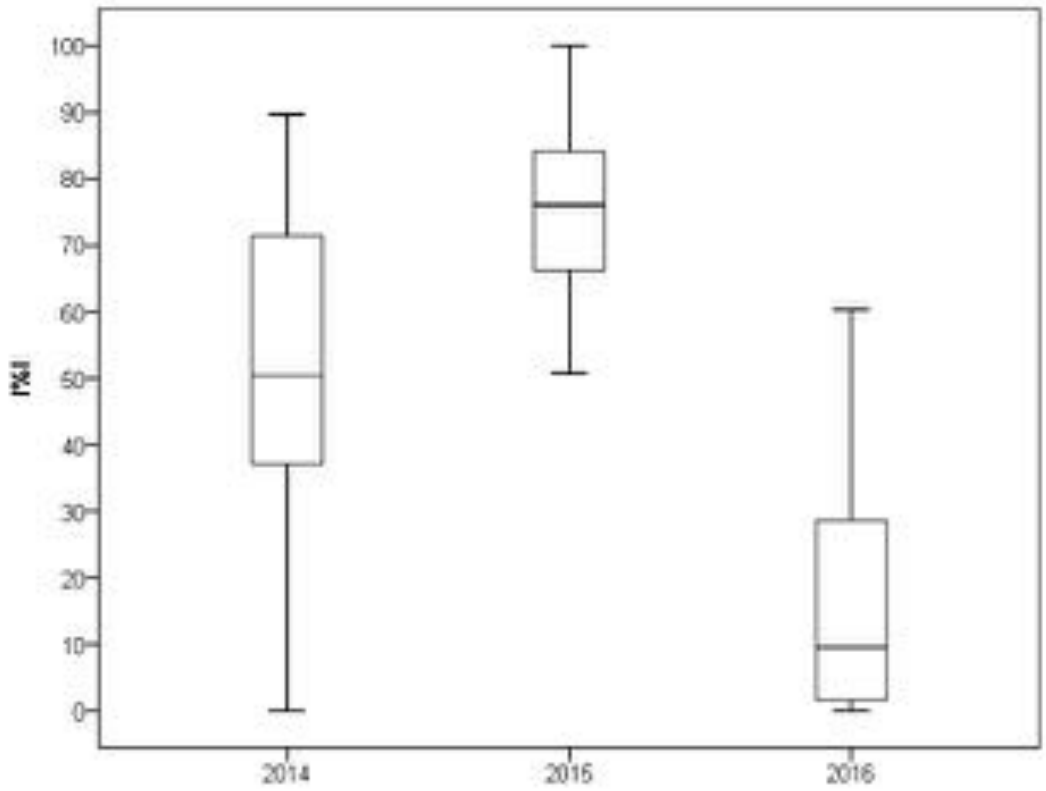
The I%I values of the susceptible reference variety in this study, 'Pinot noir', were 100, 76.2 and 84.1% respectively in 2014, 2015 and 2016, while the resistant variety, 'Bianca' recorded I%I values very low: 4. 0 and 3.2% respectively.

4.2.2 Caucasian V. vinifera subsp. sylvestris

The experimental inoculations were carried out on wild Caucasian accessions cultivated in DiSAA greenhouse.

In the Fig. 4.4 are shown the distribution of the data obtained in 2014, 2015 and 2016.

Figure 4.4 Box plot distribution of the I%I of wild Caucasian accessions from 2014 until 2016



During the first year the data were widely distributed, with I%I values ranging from 0 to 89.68 %. 20% of the wild accessions showed I%I values lower than 25%, I%I values (Table 4.7).

Table 4.7 I%I values of the Georgian accessions which resulted resistant in 2014

CULTIVAR	REGION	ACCESSION CODE	I%I
Kvetari 04	Kakheti	**13G039**	0.00
Skra 01	Kartli	**13G013**	2.38
Samebis seri 02	Kakheti	**13G047**	6.68
Unknown	Unknown	**WF 110/298**	7.86
Meneso 01	Kartli	**13G093**	15.48
Nakhiduri 06	Kartli	**13G103**	16.67
Larchvali 01	Lechkhumi	**13G030**	19.05
Unknown	Unknown	**WFKTSIA N°12**	21.43

In the second year, all the wild accessions resulted susceptible, showing I%I values higher than 50.8%.

On the contrary in 2016 most of the accessions showed a low level of disease severity susceptibility, recording 60.8% as highest value. The 75.7% of wild accessions showed a I%I values lower than 25%, (Table 4.8).

Table 4.8 I%I values of the Georgian accessions which resulted resistant in 2016

CULTIVAR	REGION	ACCESSION CODE	I%I
Skra 01	Kartli	**13G013**	0.0
Paldo 02	Kakheti	**13G032**	0.0
Tushis tbebi 02	Kakheti	**13G056**	0.0
Bagichala 16	Kartli	**13G066**	0.0
Unknown	Unknown	**13G090**	0.0
Unknown	Unknown	**13G101**	0.0
Nakhiduri 06	Kartli	**13G103**	0.0
Tskhomareti 01	Lechkhumi	**13G029**	1.6
Unknown	Unknown	**GEO W 69**	1.6
Naghomari 01	Lentekhi	**13G014**	3.2
Nakhiduri 11	Kartli	**13G105**	3.2
Larchvali 01	Lechkhumi	**13G030**	4.8
Mesamotse kvartali 03	Kakheti	**13G036**	4.8
Nakhiduri 10	Kartli	**13G104**	7.9
Kvetari 10(2)	Kakheti	**13G042**	7.9
Unknown	Unknown	**GEO W 31**	7.9
Tedotsminda 10	Kartli	**13G075**	9.5
Tedotsminda 21	Kartli	**13G085**	11.1
Zubi 01	Lechkhumi	**13G027**	12.7

CULTIVAR	REGION	ACCESSION CODE	I%I
Sabue 03	Kakheti	**13G054**	14.3
Bagichala 04+05	Kartli	**13G060**	14.3
Lamiskhevi (enageti) 01	Kartli	**13G091**	15.9
Barisakho turning 01	Kartli	**13G097**	15.9
Baisubani 01	Kakheti	**13G037**	17.5

Only two accessions confirmed the resistant behaviour in 2014 and 2016: 13G030 and 13G103 belonging to Lechkhumi and Kartli regions.

4.3 SCREENING OF MGALOBLISHVILI PROGENIES

Moreover the progeny of Mgaloblishvili N. are divided according to the type of playback.

The 'Bianca' accession, used as resistant control, showed I%I values very low in all the assays. On the contrary, the susceptible reference variety 'Pinot noir N' showed I%I higher than 50%.

4.3.1 Progeny of Mgaloblishvili:crossed with Pinot noir

The progeny of Mgaloblishvili originated by crossing with Pinot noir was assessed by experimental inoculation during 2014 and 2015. In both years all accessions resulted susceptible towards *P. viticola*, except for one individual, 271M, that in the first year of activity showed a I%I of 17.1% (Fig. 4.5).

Figure 4.5 Box plot distribution of the I%I of progeny of Mgaloblishvili originated by crossing with Pinot noir from 2014 until 2015

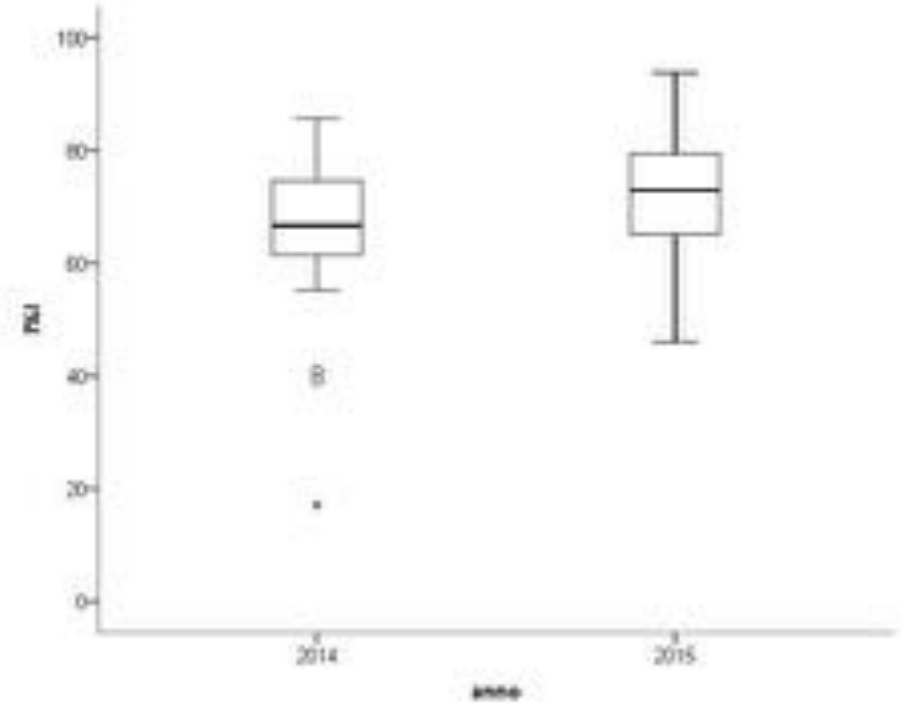
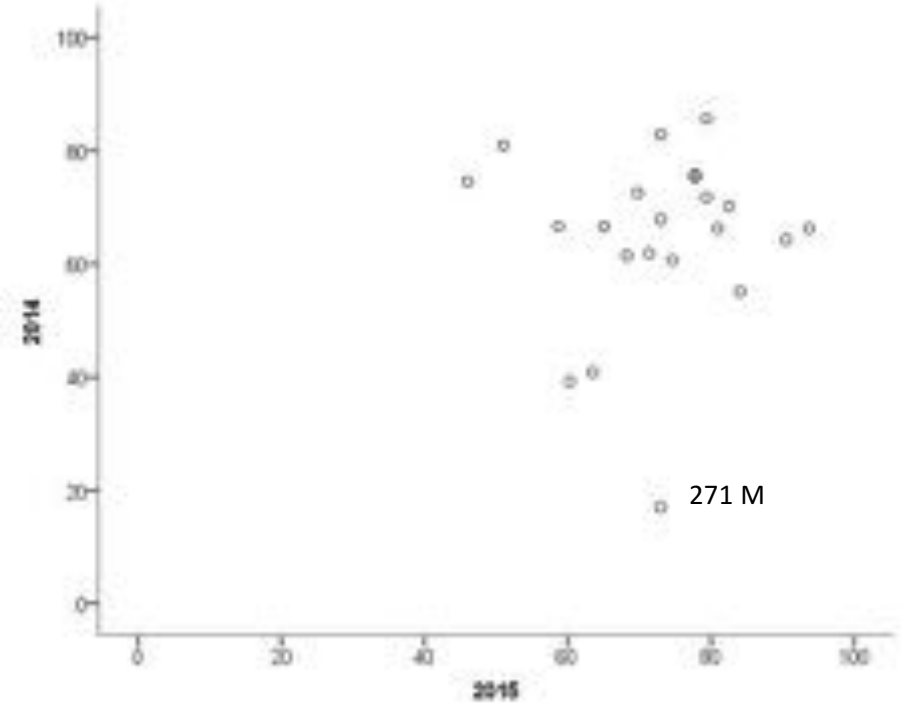


Figure 4.6 Scatter plot showing the I%I of the progeny originated by crossing with Pinot noir in 2014 and 2015

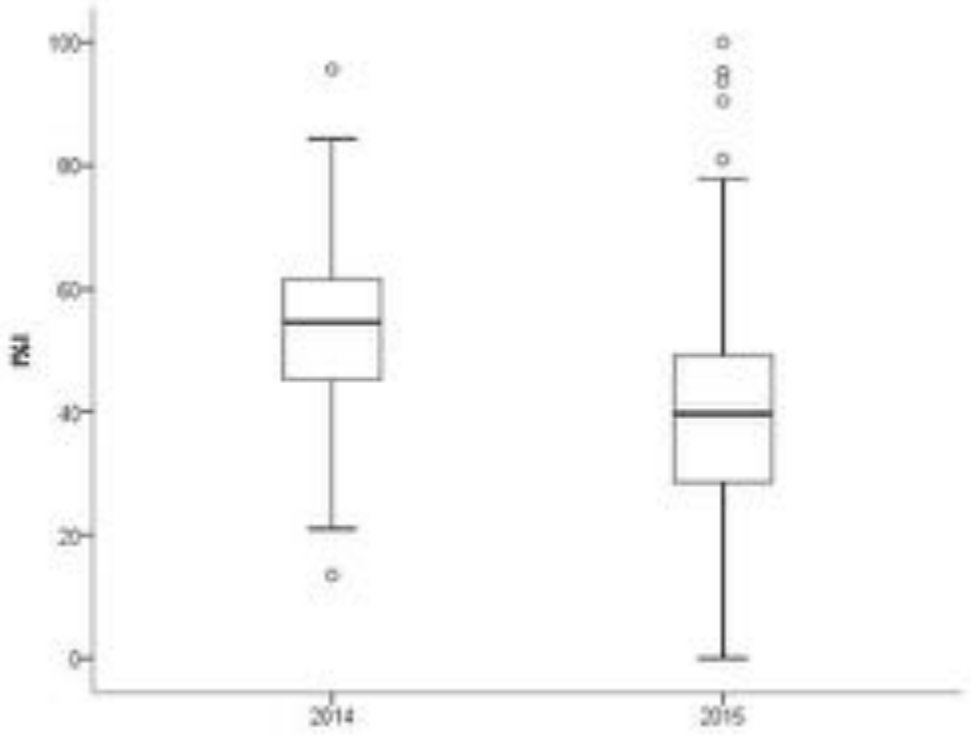


Comparing I%I obtained by experimental inoculations carried out in 2014 and 2015, all accessions in 2015 confirmed the behavior observed in 2014 (Fig. 4.6), showing a low efficiency to containment the pathogen, except for the 271M accession, which during the first year showed a good level of resistance, but in the second year resulted susceptible.

4.3.2 Progeny of Mgaloblishvili: open pollination

The experimental inoculations carried out on the progeny of Mgaloblishvili originated by open pollination showed I%I values from 13.4 to 95.6 % in 2014 (Fig. 4.6).

Figure 4.7 Box plot distribution of the I%I of progeny of Mgaloblishvili originated by open pollination from 2014 until 2015



Most of the accessions tested in 2014 resulted susceptible, being characterized by I%I values higher than 25%, but only 5 accessions resulted resistant (Table 4.9).

Table 4.9 I%I values of the accessions which resulted resistant in 2014

ACCESSION	I%I
70(AUT)	13.4
7	21
134	21
96	23.8
41	24.2

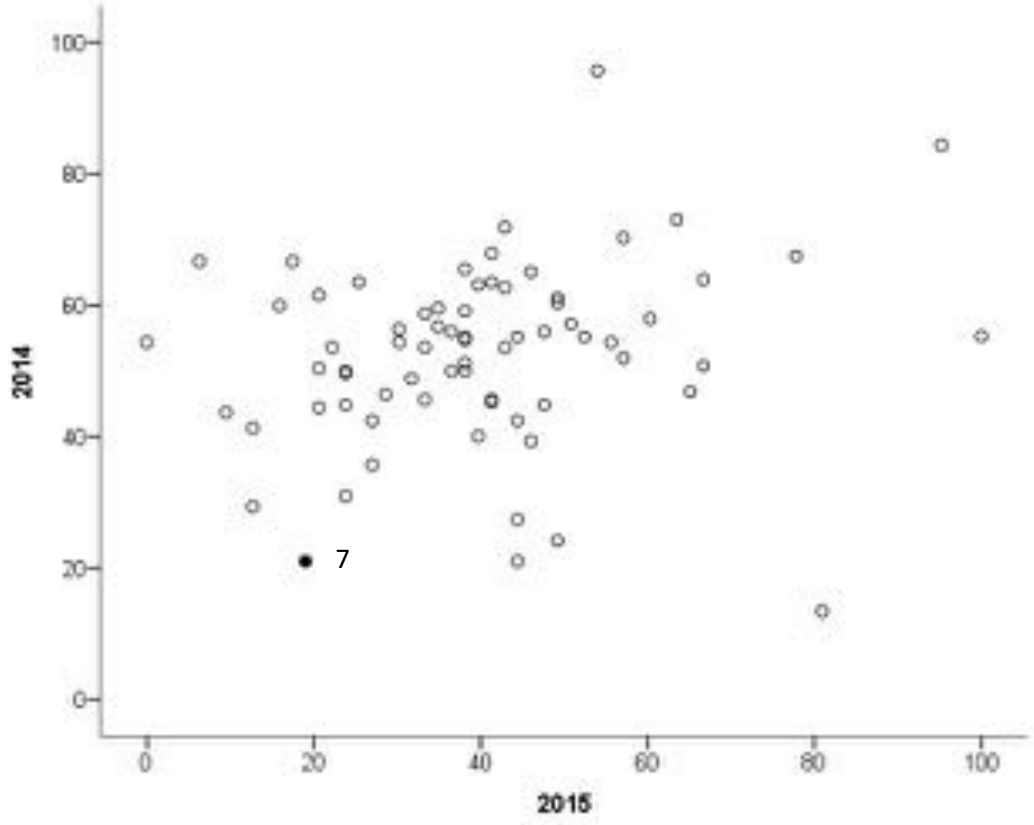
The accessions that showed a low susceptibility level in 2014 however did not confirm resistance in 2015.

In 2015 the data are more widely distributed, showing I%I values between 0 and 100%. More accessions recorded I%I values lower than 25 % (Tab. 4.10) but these accessions were susceptible in 2014. Only a single accession, the number 7, confirmed the resistant behaviour in both years, showing a I%I value of 21% in 2014 and 19% in 2015 (Fig. 4.8).

Table 4.10 I%I values of the accessions which resulted resistant in 2015

ACCESSION	I%I
34	0.0
32	6.3
80	9.5
139	9.5
22	12.7
36	12.7
155	15.9
39	17.5
7	19.0
11	20.6
19	20.6
83	20.6
160	22.2
12	23.8
46	23.8
50	23.8
106	23.8
157	23.8

Figure 4.8 Scatter plot showing the I%I of the progeny originated by open pollination in 2014 and 2015

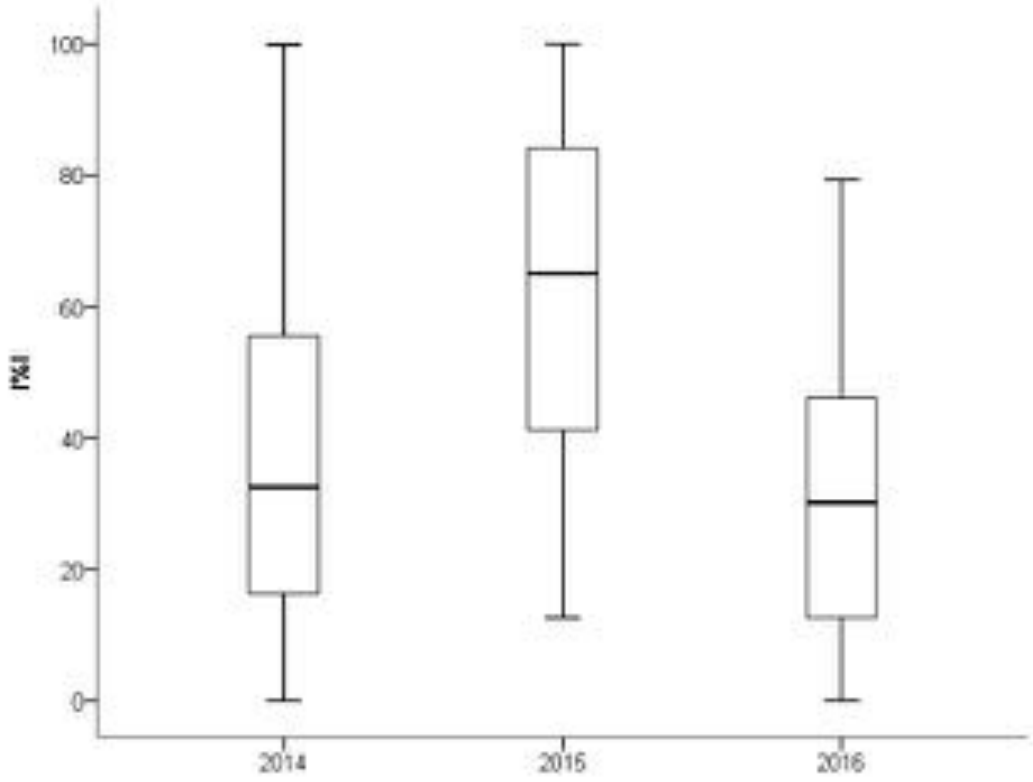


4.3.3 Progeny of Mgaloblishvili: self pollination

Finally, the behaviour of the progeny of Mgaloblishvili obtained by self pollination was evaluated.

In the 4.9 is shown the distribution of the data from 2014 until 2016.

Figure 4.9 Box plot distribution of the I%I of progeny of Mgaloblishvili originated by self pollination from 2014 until 2016



The values during the first year are comprised in an interval from 0 to 99.9%, showing a high level of variability into the progeny. During 2014 the 42.4% of the accessions shown a resistant behaviour, with I%I values lower than 26%.In Table 4.11 are listed the accessions with I%I values lower than 26%.

Table 4.11: I%I values of the accessions which resulted resistant in 2014

ACCESSION	I%I	ACCESSION	I%I	ACCESSION	I%I
89	0	8	9,9	**116**	18.4
137	0	152	10,6	**127**	19.1
9 (LIB)	0	61	11,3	**163**	19.8

ACCESSION	I%I	ACCESSION	I%I	ACCESSION	I%I
139(LIB)	1.2	96	12,0	**148 M**	19.8
122	2.1	56 (LIB)	12,6	**16 (LIB)**	20.2
153	2.8	14 (LIB)	12,7	**2 (LIB)**	20.2
124	4.3	97	12,8	**121**	20.5
19	5.0	20	13,5	**18**	22.0
113	5.0	103	13,5	**41**	22.0
74	5.7	135 M	13,5	**143**	22.0
80	5.7	151 M	14,9	**149**	22.0
62	7.1	102	15,6	**95**	22.7
186	7.1	101	16,3	**155**	22.7
128	7.8	138	16,3	**197**	24.8
59	8.5	55 (LIB)	17,5	**46**	24.8
111	8.5	60 (LIB)	17,5	**11**	25.5
151	8.5	99	17,7	**21**	25.5
65 (LIB)	8.7	125	17,7		

During the second year of experimental inoculations activity, only 8.8% of the accessions resulted resistant (Table 4.12). Most of plants showed I%I values higher than 25%.

Table 4.12: I%I values of the accessions which resulted resistant in 2015

ACCESSION	I%I	ACCESSION	I%I
197	12.7	**30 (LIB)**	22.2
74 (LIB)	12.7	**73 (LIB)**	22.2
84	15.9	**114**	23.8
147 (LIB)	17.5	**78 (LIB)**	23.8
96	20.6	**9**	25.4
94 (LIB)	20.6	**110**	25.4
149	22.2	**86**	25.5

In 2016, 39.6% of the accessions showed a good level of resistance against *P. viticola* (Table 4.13).

Table 4.13: I%I values of the accessions which resulted resistant in 2016

ACCESSION	I%I	ACCESSION	I%I	ACCESSION	I%I
15	0.0	**19**	9.5	**176**	14.3
97	0.0	**83**	9.5	**25(LIB)**	14.3
145(LIB)	0.0	**188**	9.5	**106**	17.5
114	3.2	**186**	11.1	**148M**	17.5
158(LIB)	3.2	**199**	11.1	**78(LIB)**	17.5
40	4.8	**56(LIB)**	11.1	**85(LIB)**	19.0
153	4.8	**135**	12.7	**67**	20.6
155M	4.8	**150(LIB)**	12.7	**115**	22.2
157M	4.8	**18**	12.7	**9(LIB)**	22.2
74(LIB)	6.3	**43**	12.7	**57**	25.4
16	7.9	**113**	12.7	**18(LIB)**	25.4
45	7.9	**181**	12.7	**94(LIB)**	25.4
55	7.9	**66(LIB)**	12.7	**46**	25.4
82	7.9	**124**	14.3	**122**	25.4

During the three years of experimental inoculations each accession showed a different behavior. The accessions which resulted resistant during a single year did not reconfirm the resistant behavior in the other two years. In the figure 4.10 are shown the I%I values that each accession recorded during the three years. The bars indicate the distance of the values obtained in the different years.

It is possible to observe that each accession showed a large variability in the level of resistance, except for two accessions: 124 and 147 (LIB). These two accessions recorded the lowest I%I values in 2014, 2015 and 2016 (Table 4.14).

Figure 4.10 Scatter plot with bars that indicate the distance of the values of the progeny obtained by self pollination in 2014, 2015 and 2016. showing the I%I of the progeny originated by self pollination which result resistant

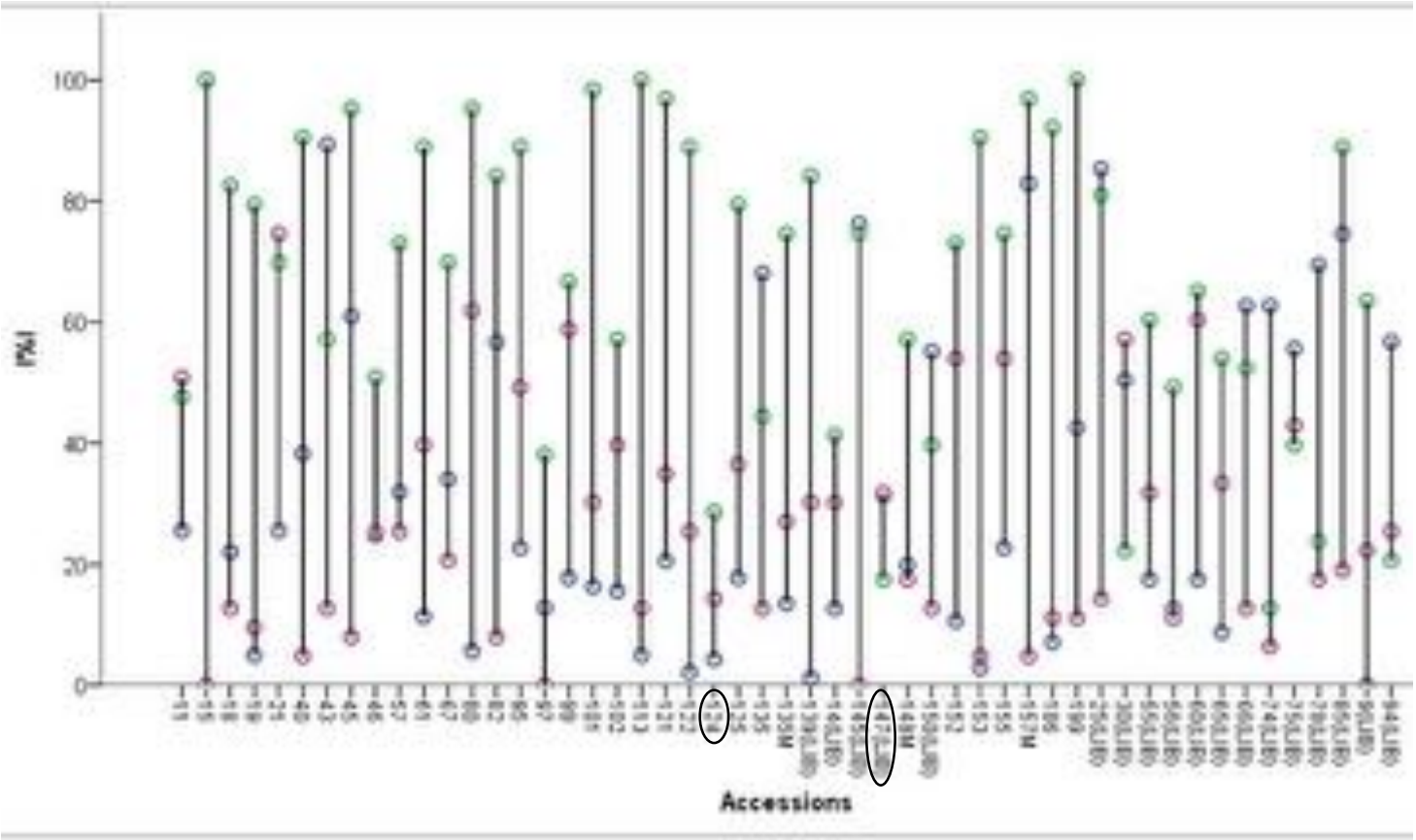


Table 4.14: I%I values of the accessions that confirmed the resistant behavior in 2014, 2015 and 2016.

ACCESSION	2014 I%I	2015 I%I	2016 I%I
124	4.3	28.6	14.3
147 (LIB)	31.3	17.5	31.7

The accession 124 showed a good level of resistance, in particular in 2014 and 2016, whereas 147 (LIB) resulted medium resistant in 2014 and 2016 and resistant in 2015.

4.4 HISTOLOGICAL ANALYSIS

Both the callose deposition by the plant and the pathogen structures were investigated at 1, 2, 3, and 6 dai (day after inoculation) on Pinot noir, Mgaloblishvili and Bianca.

4.4.1 24 hours after inoculation

During the investigation carried out on Pinot noir, susceptible towards *P. viticola*, and Mgaloblishvili, it was observed a positive infection response by the pathogen just after 24 hours after the inoculation. It was possible to notice, in proximity of the stomata, encysted zoospores, from whose arose the germ tube which penetrated through the stomata and, into the substomatal cavity, forming a vesicular structure, named substomatal vesicle (Fig. 1- a and 2- a), from whom the mycelium originates.

On Bianca, the resistant varieties, in the first infection stages, callose

depositions were observed on the cells surrounding the substomatal cavity immediately underneath the germinating zoospore. The resistant reactions therefore likely occurr early in the colonization process and seem to limit the pathogen growth instead of completely blocking it.

Colonization patterns in resistant genotype is associated with numerous changes in both the structural and chemical leaf characteristics, due to the activation of complex defense responses which eventually cause the necrotic lesions and the reduced colonization (Toffolatti *et al.,* 2012).

This response is leaded by the activation of several defence mechanisms that all established the hypersensitive response (HR), including the production of antimicrobial metabolites and proteins and, at the cell wall level, thickenings, callose appositions in the paramural space and accumulation of phenolic compounds and reactive oxygen species. (Fig. 3- a).

4.4.2 48 hours after inoculation

At 48 hours after inoculation, the primary hypha with haustoria, recognizable from the brightly fluorescent neck covered by callose, started branching inside the leaf tissues in Pinot noir and Mgaloblishvili (Fig. 1- b and 2- b).

On Bianca, are visible evident differences compared to Pinot noir and Mgaloblishvili: the hyphal structures are not visible, but the reactions typical of hypersensitive response (fluorescent reaction) are visible on the stomata (Fig. 3- b).

4.4.3 72 hours after inoculation

72 hours after, the differences were visible among Pinot noir and Mgaloblishvili. In fact in Pinot noir (Fig. 1- c) *P. viticola* developed a linear mycelium with some haustoria, and it appears active and alive. The outline of the hyphae appeared well defined. On the contrary, the structure of mycelium in Mgaloblishvili is altered: *P. viticola* produced multi-branched hyphae (Fig. 2- c) with numerous haustoria. The outline of the mycelium do not appear defined, and the hyphae did not seem viable.

No traces of pathogen structures were visible in Bianca leaves.

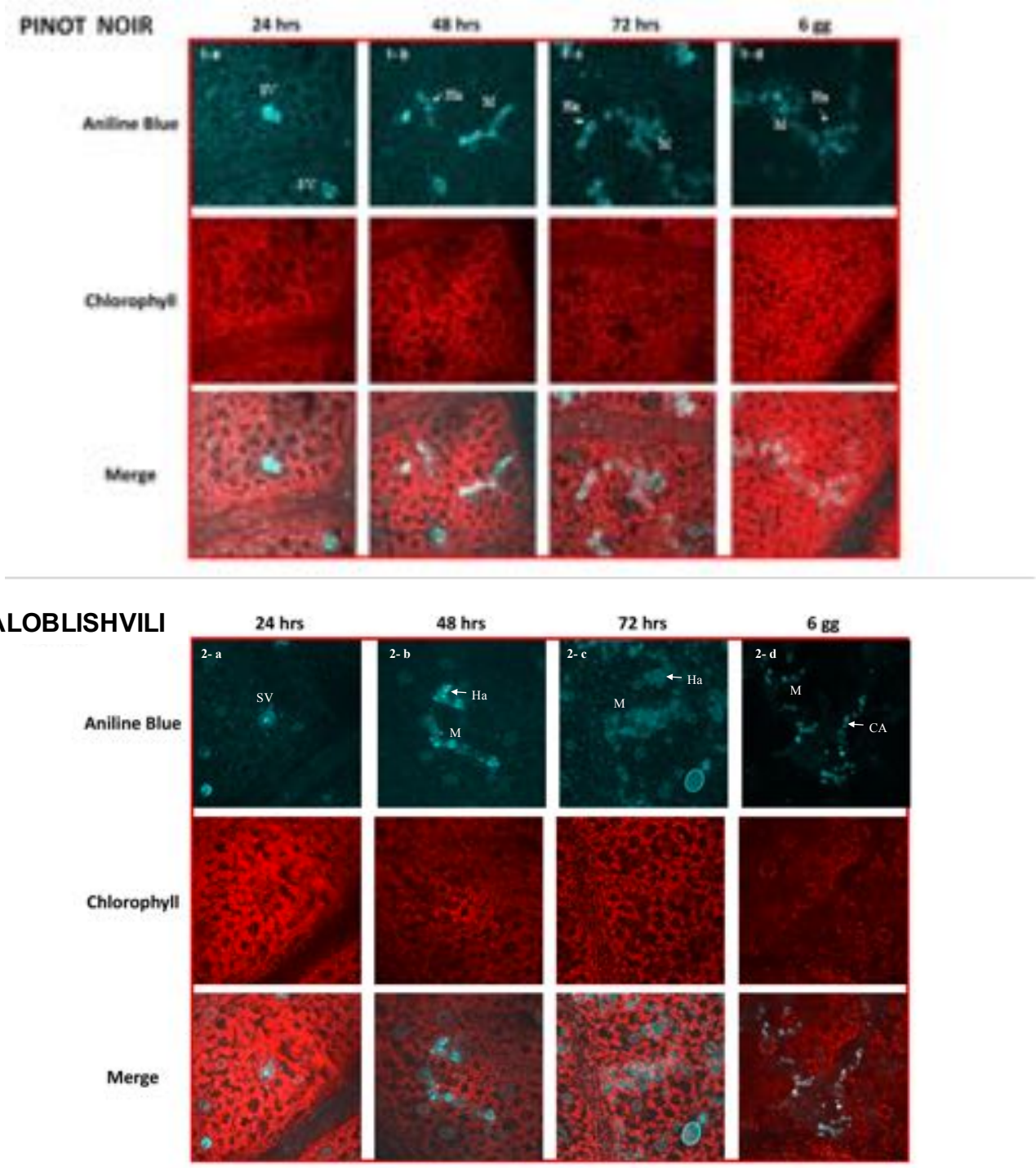
4.4.4 6 days after inoculation

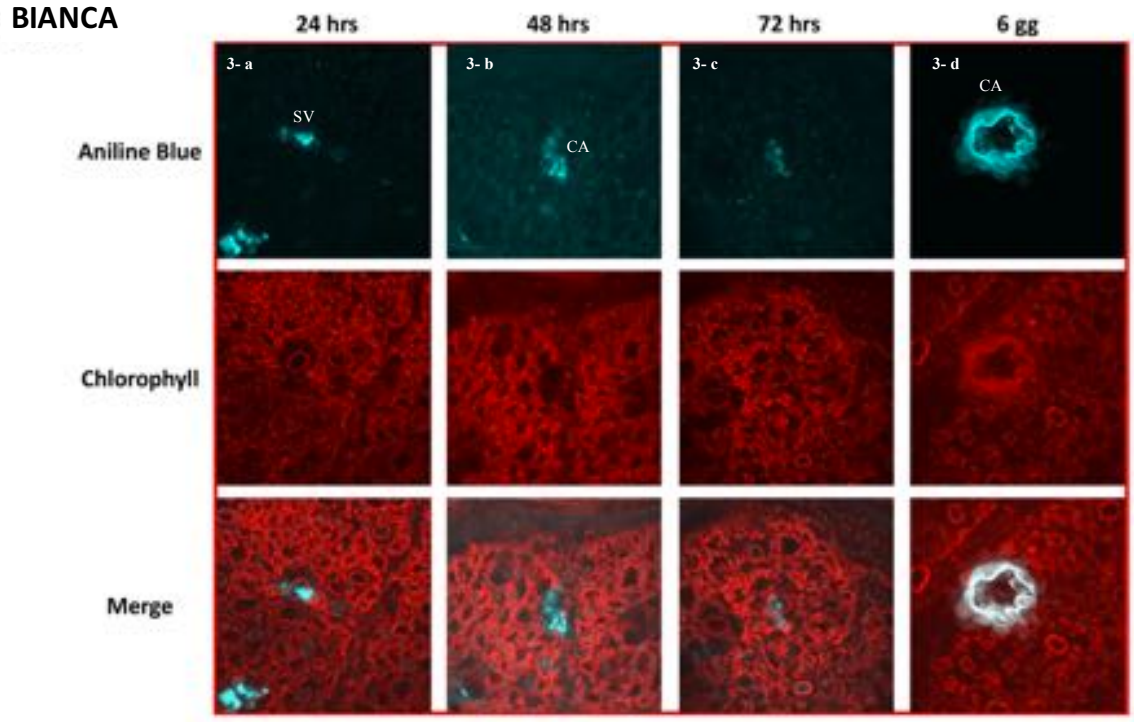
6 days after inoculation, *P. viticola* showed an extensive growth in the leaf, with regular vegetative and reproductive structures, with emission of a single sporangiophore bearing sporangia from each stomata (not shown) (Fig. 1- d).

In Mgaloblishvili the mycelium appeared with no regular diameter (Fig. 2- d). It was observed (not showed in the pictures) the formation of sterile, hyper-branched sporangiophores through stomata. Moreover the mycelium developed in Mgaloblishvili is characterized by callose apposition probably synthesized by the pathogen (the callose was formed into the mycelium).

In bianca the hypersensitive reaction was affirmed evidently.

Figure 4.11: Time course of colonization of Pinot noir, Mgaloblishvili and Bianca by *P. viticola*





SV = substomatal vesicle

M = mycelium

Ha = haustorium

CA = callose apposition

4.5 COMPARATIVE EVALUATION OF AGGRESSIVENESS

The disease severity evaluated inoculating different *P. viticola* samples on cv Bianca, (resistant control), Pinot noir (susceptible control) and Mgaloblishvili, was used to evaluate the level of aggressiveness of the pathogen. At the same time, it was possible to investigate the behavior of Mgaloblishvili in relation with the aggressiveness level and how this can modulate the response of the plant.

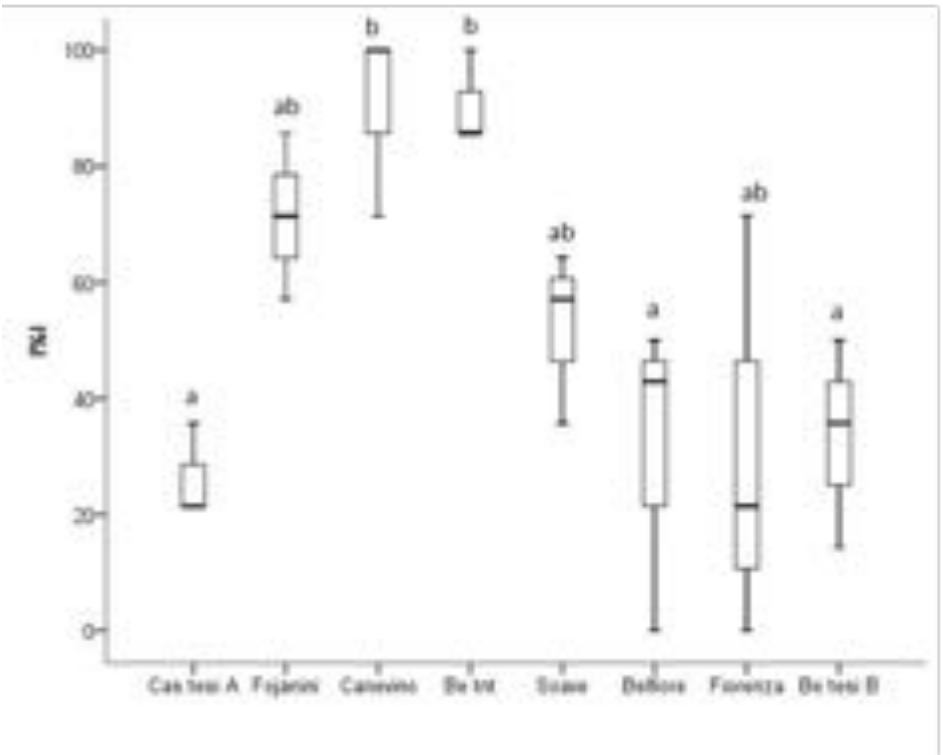
4.5.1 Comparison among P. viticola strains

The statistical analysis has been carried out among inocula, to analyse the different aggressiveness levels on Pinot noir for each *P. viticola* population and the different behaviour of Mgaloblishvili, in relation with the sample used in the experimental inoculation.

<u>*Pinot noir*</u>

Based on Kruskal-Wallis test significant differences were observed among *P. viticola* samples on Pinot noir (H=16.4; df=7; *P*=0.02) (Fig. 4.12). B, D and G, resulted the most aggressive strains.

Figure 4.12: Box plot distribution of the I%I of Pinot noir in relation with the different inocula.



<u>*Mgaloblishvili*</u>

In the Fig. 4.13 are shown the distribution of the I%I values recorded by Mgaloblishvili inoculated with the different inocula. Great differences in the response to *P. viticola* were observed on Mgaloblishvili in relation with the aggressiveness of the pathogens (H=21.1; df=7; P=0.004) (Fig. 4.18), inferring that the resistance level of this cultivar is modulated by the aggressiveness level of the pathogen.

Figure 4.13: Box plot distribution of the I%I of Mgaloblishvili in relation with the different inocula. The same letter indicate not significantly differences at *P* = 0.05 level of probability.

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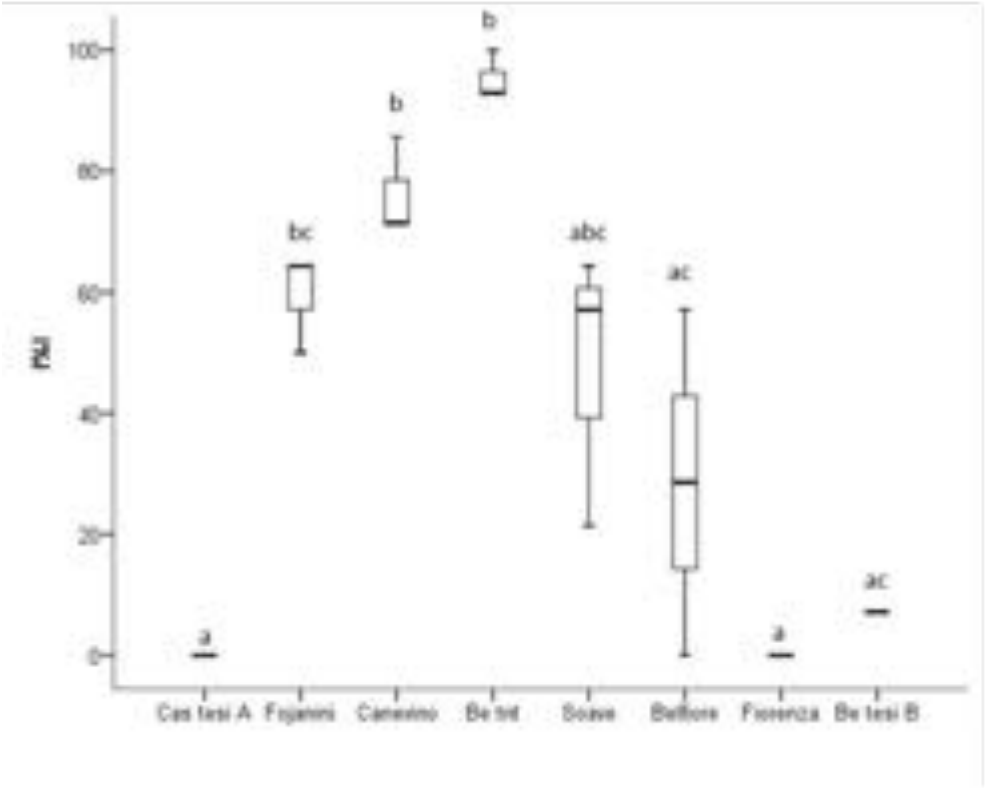


4.5.2 Aggressiveness of P. viticola strains

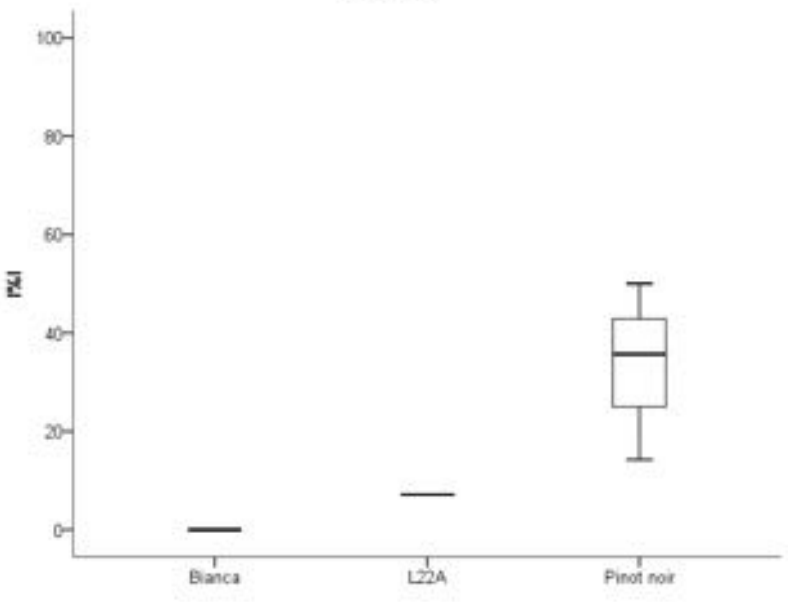
The aggressiveness level of the different samples was estimated referring to the I%I observed on Pinot noir. High I%I values on the susceptible control indicated high aggressiveness level of the pathogen. The I%I values on Pinot noir were oscillated between 26.2 and 90.5%, underling a high difference in the aggressiveness level of the pathogen.

a) Strain A

The inoculum A, belonging to a vineyard located in Santa Maria della Versa (PV), showed a medium aggressiveness level, as it can be inferred by the I%I value recorded on Pinot noir (33.3%) (Fig. 4.14B). According to the statistical analysis significant differences among the I%I recorded on the three cultivars were obtained (H=7.7; df=2; *P*=0.021). Mgaloblishvili did not show significant differences with Bianca, revealing a good level of resistance. Both the cultivars resulted significantly different from Pinot noir (Fig. 4.14).

Figure 4.14: Box plot distribution of the I%I of Bianca, Mgaloblishvili and Pinot noir, inoculated with the strain A (A) and the I%I values of each plant. The average percentages followed by the same letter are not significantly different at α = 0.05 level of probability (B).

A



B

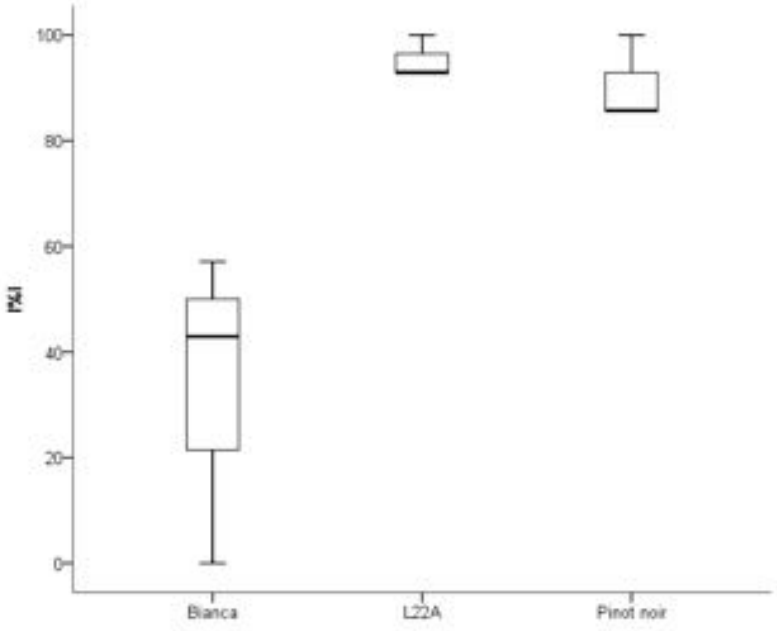
CULTIVAR	I%I
BIANCA	0.0 a
MGALOBLISHVILI	7.1 a
PINOT NOIR	33.3 b

b) <u>Strain B</u>

The inoculation carried out with B, sampled in a second vineyard located at Santa Maria della Versa (PV), revealed a seriously high aggressiveness level, as it can be proved by the I%I value on Pinot noir (90.5%) and, in particular, by the relatively high I%I value of Bianca (33.3%) (Fig. 4.15B). Based on statistical analysis (H=5.9; df=2; *P*=0.04) significant differences were observed comparing the cultivars. Mgaloblishvili (95,2%) resulted significantly different from Bianca (33,3%), and it did not show significant differences with Pinot noir (Fig. 4.15).

Figure 4.15: Box plot distribution of the I%I values recorded on Bianca, Mgaloblishvili and Pinot noir, following inoculation with the strain B (A) and the I%I values of each plant (B). The average percentages followed by the same letter are not significantly different at $\alpha = 0.05$ level of probability (B).

A



B

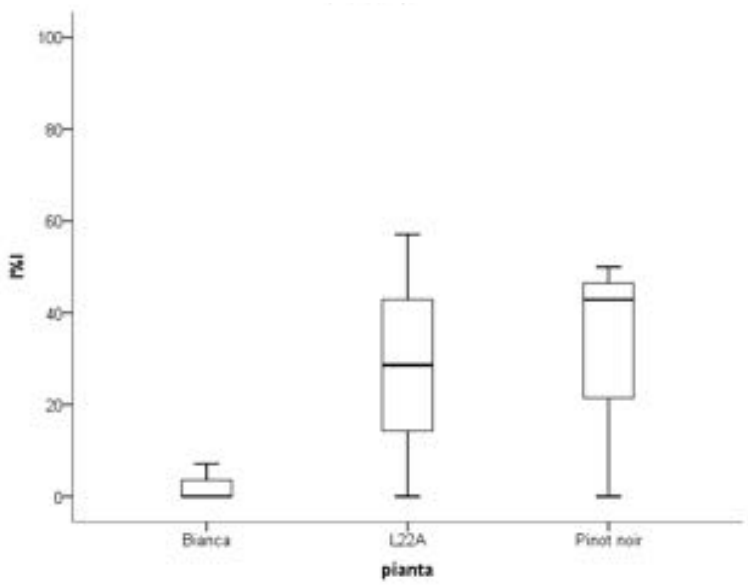
CULTIVAR	I%I
BIANCA	33.3 a
MGALOBLISHVILI	95.2 b
PINOT NOIR	90.5 b

c) <u>Strain C</u>

The *P. viticola* strain, collected in Belfiore (VR), was characterized by a medium aggressiveness level: a low I%I value (31%) was recorded on Pinot noir (Fig. 4.16B). According to Kruskal-Wallis test, no significant differences could be observed among cultivars (H=1.8; df=2; *P*=0.4). Bianca, Mgaloblishvili and Pinot noir show, indeed, I%I values very low (Fig 4.16).

Figure 4.16: Box plot distribution of the I%I values recorded on Bianca, Mgaloblishvili and Pinot noir, following inoculation with the strain C (A) and average I%I values of each plant (B). The percentages followed by the same letter are not significantly different at $\alpha = 0.05$ level of probability (B).

A



B

CULTIVAR	I%I
BIANCA	2.4 a
MGALOBLISHVILI	28.6 a
PINOT NOIR	31.0 a

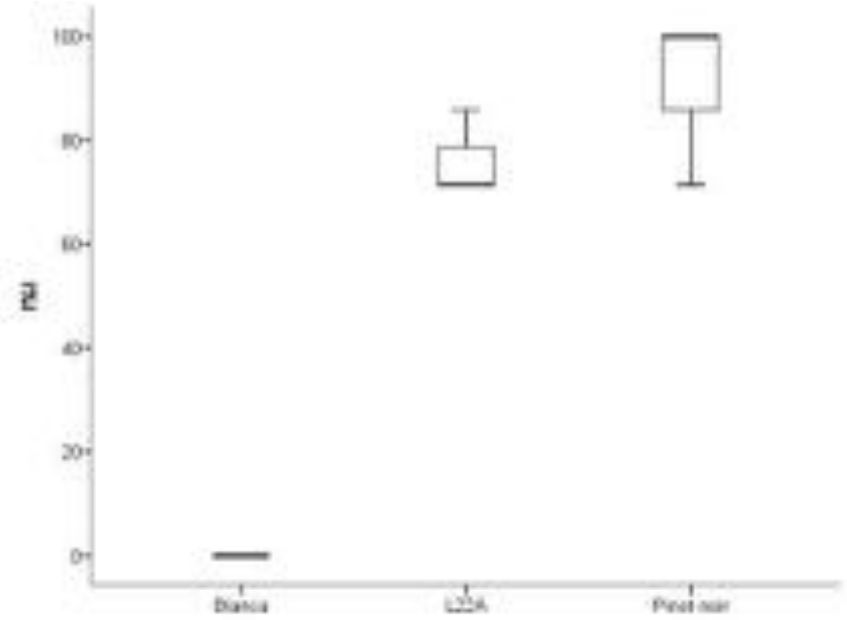
d) Strain D

The inoculum collected in Canevino (PV), showed a high aggressiveness level: Pinot noir in fact revealed a I%I value of 90.5% (Fig. 4.17).

Significant differences were obtained by the statistical analysis (H=6.4; df=2; *P*=0.04). Mgaloblishvili recorded a I%I value of 76.2%, a value analogous to that of Pinot noir. Mgaloblishvili was significantly different from Bianca which showed the absence of sporulation (Fig. 4.12).

Figure 4.17: : Box plot distribution of the I%I values recorded on Bianca, Mgaloblishvili and Pinot noir, following inoculation with the strain D (A) and average I%I values of each plant (B). The percentages followed by the same letter are not significantly different at $\alpha = 0.05$ level of probability (B).

A



B

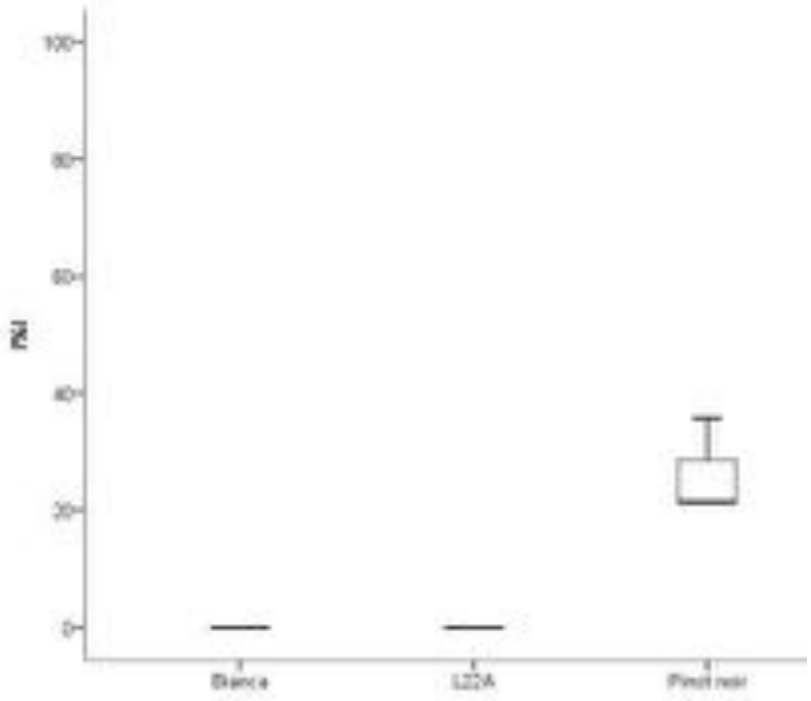
CULTIVAR	I%I
BIANCA	0.0 a
MGALOBLISHVILI	76.2 b
PINOT NOIR	90.5 b

e) Strain E

The inoculation carried out with Cas tesi A, belonging to Casarsa della Delizia (PN), highlighted significant differences among the plants (H=7.7; df=2; *P*=0.021). The inoculum showed low aggressiveness level, proved by the low infection resulted in Pinot noir (26.2%) (Fig. 4.18). No infection resulted on Mgaloblishvili and Bianca which both resulted statistically different from Pinot noir (Fig. 4.13).

Figure 4.18: Box plot distribution of the I%I values recorded on Bianca, Mgaloblishvili and Pinot noir, following inoculation with the strain D (A) and average I%I values of each plant (B). The percentages followed by the same letter are not significantly different at $\alpha = 0.05$ level of probability (B).

A



B

CULTIVAR	I%I
BIANCA	0.0 a
MGALOBLISHVILI	0.0 a
PINOT NOIR	26.2 b

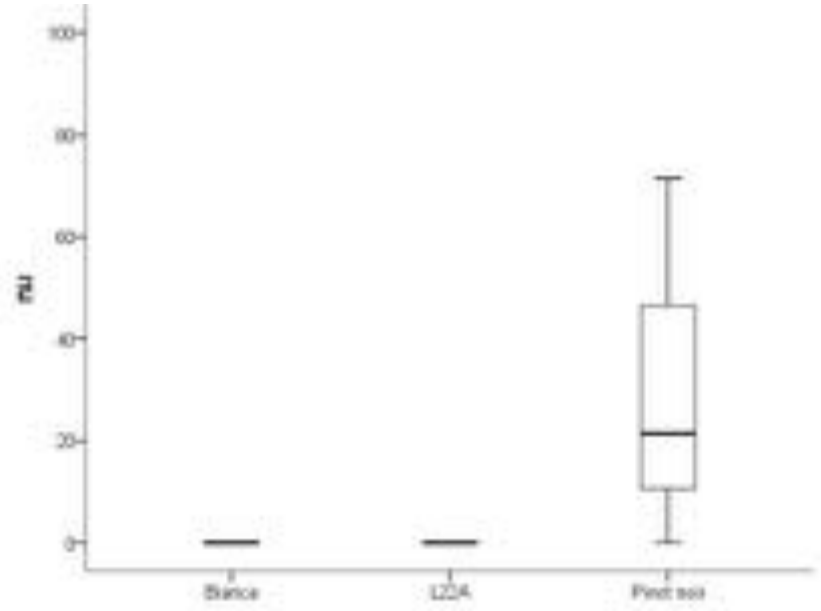
f) Strain F

Based on statistical analysis no significant differences were found among the cultivars inoculated with the F strains, sampled at Piateda (SO) (H=4.5;df=2; *P*=0.10). On Bianca and Mgaloblishvili no sporulation was

observed on the leaf discs, Pinot noir recorded a I%I value of 31%, underlying medium level of aggressiveness of the pathogen (Fig. 4.19).

Figure 4.19: Box plot distribution of the I%I values recorded on Bianca, Mgaloblishvili and Pinot noir, following inoculation with the strain F (A) and average I%I values of each plant (B). The percentages followed by the same letter are not significantly different at $\alpha = 0.05$ level of probability (B).

A



B

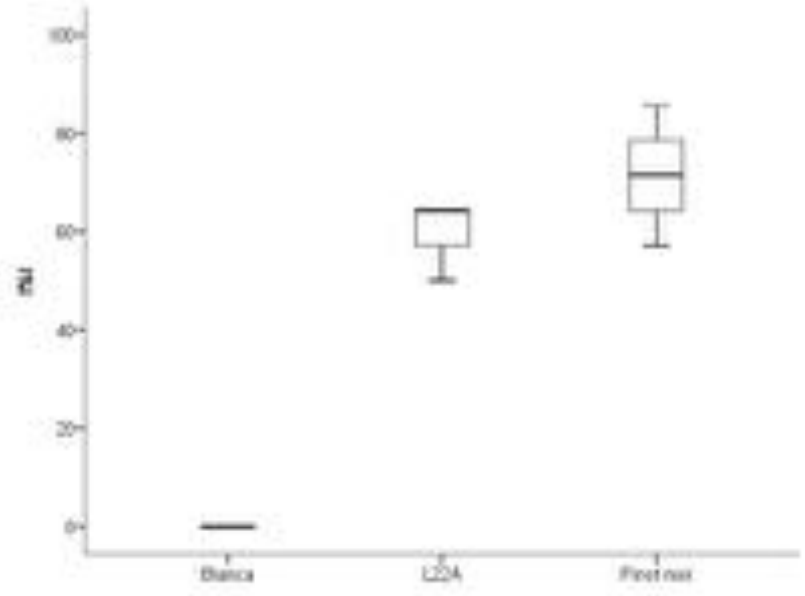
CULTIVAR	I%I
BIANCA	0.0 a
MGALOBLISHVILI	0.0 a
PINOT NOIR	31.0 a

g) Strain F

The inoculum collected in Sondrio (SO), showed a high aggressiveness level, resulting in a I%I value on Pinot noir of 71.4% (Fig. 4.20). Significant differences were obtained among Bianca and Mgaloblishvili, whereas Mgaloblishvili showed a behavior similar to that Pinot noir (H=6.2; df=2; *P*=0.045) (Fig. 4.20).

Figure 4.20: Box plot distribution of the I%I values recorded on Bianca, Mgaloblishvili and Pinot noir, following inoculation with the strain F (A) and average I%I values of each plant (B). The percentages followed by the same letter are not significantly different at $\alpha = 0.05$ level of probability (B).

A



B

CULTIVAR	I%I
BIANCA	0.0 a
MGALOBLISHVILI	59.5 b
PINOT NOIR	71.4 b

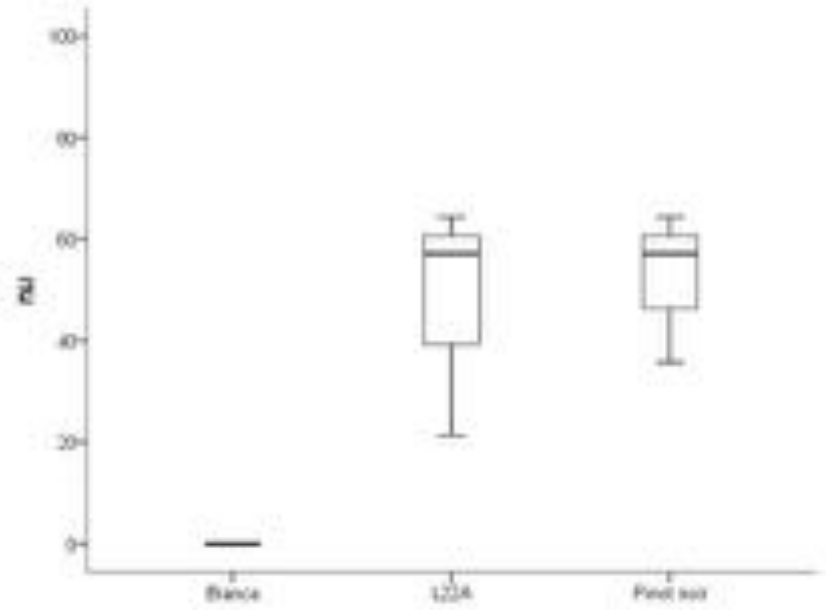
h) <u>Strain H</u>

The *P. viticola* samples belonging to Soave (VR), were characterized by a medium aggressiveness level, in fact Pinot noir recorded a I%I value of

52.4% (Fig. 4.21B). All the cultivars resulted statistically similar (H=5.7; df=2; *P*=0.058) (Fig 4.21).

Figure 4.21: Box plot distribution of the I%I values recorded on Bianca, Mgaloblishvili and Pinot noir, following inoculation with the strain H (A) and average I%I values of each plant (B). The percentages followed by the same letter are not significantly different at $\alpha = 0.05$ level of probability (B).

A



B

CULTIVAR	I%I
BIANCA	0.0 a
MGALOBLISHVILI	47.6 a
PINOT NOIR	52.4 a

4.5.2 Quantification of sporangia

Based on the results of ANOVA, the number of sporangia, a fitness component of *P. viticola* strains, varied among the cultivar inoculated:

Bianca, Mgaloblishvili and Pinot noir.

Significant differences were observed in all cases ($F > 14,2$; df= 2-8; $P < 0.005$), except for the C inoculum ($F < 4.2$; df= 2-8; $P > 0.072$) which did not reveal significant differences also for the I%I, and H, whose I%I values resulted in Mgaloblishvili and Pinot noir were not very high (Table 4.15).

Table 4.15: Average values of sporangia x10^3 /cm^2 of the accessions and results of statistical analysis*

Inoculum	**Cultivar**		
	Pinot	**Bianca**	**Mgaloblishvili**
A	50,0	0,0	16,2
B	157,6	0,6	135,0
C	79,1	0,1	50,0
D	217,0	0,0	128,5
G	170,2	0,0	99,1
H	96,5	0,0	64,2

* Mean values within the column followed by the same letter are not significantly different at 0.05 significance level.

4.6 GENETIC STRUCTURE OF P. viticola POPULATION

96 *P. viticola* strains were genotyped for 21 microsatellites loci. During the analysis ten microsatellites were not considered because of the high percentage of missing data: PV31, PV7, PV103, PV74, PV138, PV137, PV126, PV76, PV67, PV140.

The number of alleles, the allele size range, the observed heterozygosity (H_O), the unbiased expected heterozygosity (H_E) and the departure from the Hardy-Weinberg equilibrium (*HWE*) are listed in the table 4.16.

Of the 21 examined, 19 microsatellites were polymorphic and exhibited a number of alleles ranging from 2 (PV39, PV104 and PV142) to 6 (ISA and PV31). The microsatellites PV87 and PV184 were monomorphic. The H_E, also known as *Nei*'s genetic diversity (Nei, 1973), was between 0.0 for the monomorphic loci (PV87 and PV134) and 0.66 (PV14).

Among the 19 polymorphic microsatellite markers, 10 showed a deviation from the Hardy-Weinberg equilibrium. Six loci (PV31, PV16, PV65, PV104, PV142 and PV127) displayed strong heterozygote deficiency (P<0.001) compared to what would be expected under Hardy–Weinberg equilibrium (Table 4.16).

Table 4.16: Number of alleles (N_a), size range, observed (H_O) and unbiased expected (H_E) heterozygosity and significant deviation from the Hardy-Weinberg equilibrium (*HWE*) of the 21 examined microsatellites in the *P. viticola* strains.

Locus	***MD%***	N_a	**Allele size range (bp)**	H_O	H_E	***HWE***
PV14	3.0	3	120-124	0.82	0.66	*
ISA	1.0	6	112-138	0.76	0.60	*
PV17	2.0	4	142-148	0.80	0.63	**
PV39	3.0	2	175-177	0.08	0.08	ns

Locus	*MD%*	N_a	Allele size range (bp)	H_O	H_E	*HWE*
PV31	3.0	6	237-242	0.52	0.44	***
PV16	2.0	4	245-250	0.47	0.39	***
PV91	12.1	3	142-146	0.59	0.50	ns
PV147	7.1	5	195-219	0.57	0.47	ns
PV148	6.1	3	126-137	0.20	0.22	ns
PV93	8.1	3	148-152	0.49	0.42	ns
PV141	6.1	3	189-192	0.57	0.45	*
PV65	7.1	3	194-198	0.08	0.51	***
PV104	10.1	2	322-324	0.01	0.10	***
***PV87**	10.1	1	154	0.00	0.00	
PV88	11.1	2	204-206	0.16	0.19	ns
PV83	11.1	3	238-242	0.09	0.09	ns
PV142	7.1	2	209-211	0.68	0.46	***
***PV134**	7.1	1	224	0.00	0.00	
PV139	10.1	3	131-135	0.07	0.07	ns
PV127	13.1	4	216-221	0.22	0.22	***
PV101	9.1	3	262-266	0.41	0.45	ns

MD%: mean proportion of missing data over loci.

*Monomorphic

Hardy-Weinberg disequilibrium: *ns*=not significant. * P<0.05. ** P<0.01.

*** P<0.001

There are no strains with identical alleles at all loci. Therefore, all the strains represent a distinct multilocus genotype. There are only 2 couples of samples that show a low level of genotypic diversity. The strains 26 and 40 and the strains 27 and 60 are different for only 2 loci and identical for 19 loci (Table 4.17). Furthermore,10 couples of strains differ only for 3 loci (Table 4.18) (The samples name are listed in Material and Methods).

Table 4.17: List of sample pairs that differ for only 2 loci.

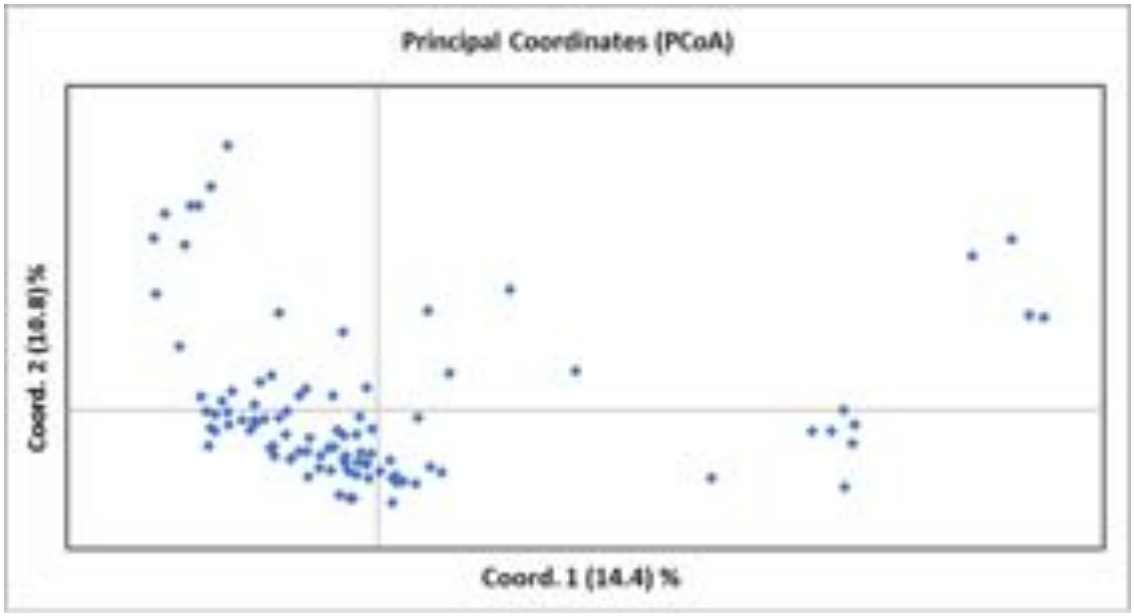
Sample pair	Strain	Location
1	26	Tuscany
	40	Lombardy
2	27	Tuscany
	60	Tuscany

Table 4.18: List of sample pairs that differ for only 3 loci.

Sample pair	Strain	Location	Couple	Sample	Location
1	1	Tuscany	6	29	Tuscany
	27	Tuscany		60	Tuscany
2	23	Tuscany	7	27	Tuscany
	27	Tuscany		61	Liguria
3	27	Tuscany	8	60	Tuscany
	29	Tuscany		61	Liguria
4	26	Tuscany	9	47	Sicily
	31	Lombardy		76	Sicily
5	51	Tuscany	10	74	Piedmont
	56	Tuscany		79	Umbria

Principal coordinates analysis (PCoA) revealed a considerable subdivision of the strains in different groups (Figure 4.22).

Figure 4.22: PCoA plot conducted with Genalex 6.501 on pairwise genetic distances between 96 *P. viticola* strains.



Analysis on strains clustered by regions

The strains were subdivided by regions to evaluate if the subdivision observed in the PCoA analysis was consistent with a geographical differentiation. Based on the region of origin (*i.e.* Lombardy, Piedmont, Tuscany and Veneto), the *P. viticola* strains were divided in four groups. The other regions were excluded from the dataset because the genotypes did not reach the minimum number to perform the analysis.

In Table 4.19 are shown the strains characterized by private alleles. The private alleles are alleles that are found only in a single group. The frequencie of each private allele was, in general, very low.

Table 4.19: Strains of *P. viticola* with private alleles.

Region	Locus	Allele
Tuscany	ISA	133
Tuscany	PV17	148
Tuscany	PV31	241
Tuscany	PV91	146
Tuscany	PV148	126
Tuscany	PV83	240
Tuscany	PV127	216
Tuscany	PV127	221
Veneto	PV16	250
Veneto	PV147	219

The analysis of molecular variance (AMOVA) was calculated on the four clusters to evaluate the existence of significant genetic differentiations (F_{ST}) caused by geographic distance (Table 4.20).

The AMOVA showed that 100% of the total of variation was distributed within clusters and no variation was explained by differences among groups. No significant differentiation was observed among the populations (P =0.58) with a total F_{ST} value of 0.0, indicating that there are not differences among individuals grouped by region. F_{ST} is directly related to the variance in allele frequency among clusters and, conversely, to the degree of resemblance among individuals within groups. If F_{ST} is small, it means that the allele frequencies within each cluster are similar; if it is large, it means that the allele frequencies are different.

Table 4.20: Analysis of molecular variance among and between groups of *P. viticola* measured by F_{ST}.

Source	df	SS	MS	%	F_{ST}	*P*
Among Pops	3	28.008	9.336	0%	0.0	0.58
Within Pops	73	731.408	10.019	100%		
Total	76	759.416		100%		

No significant genetic differentiation were detected among *P. viticola* strains (in all cases F_{ST}>0.05).

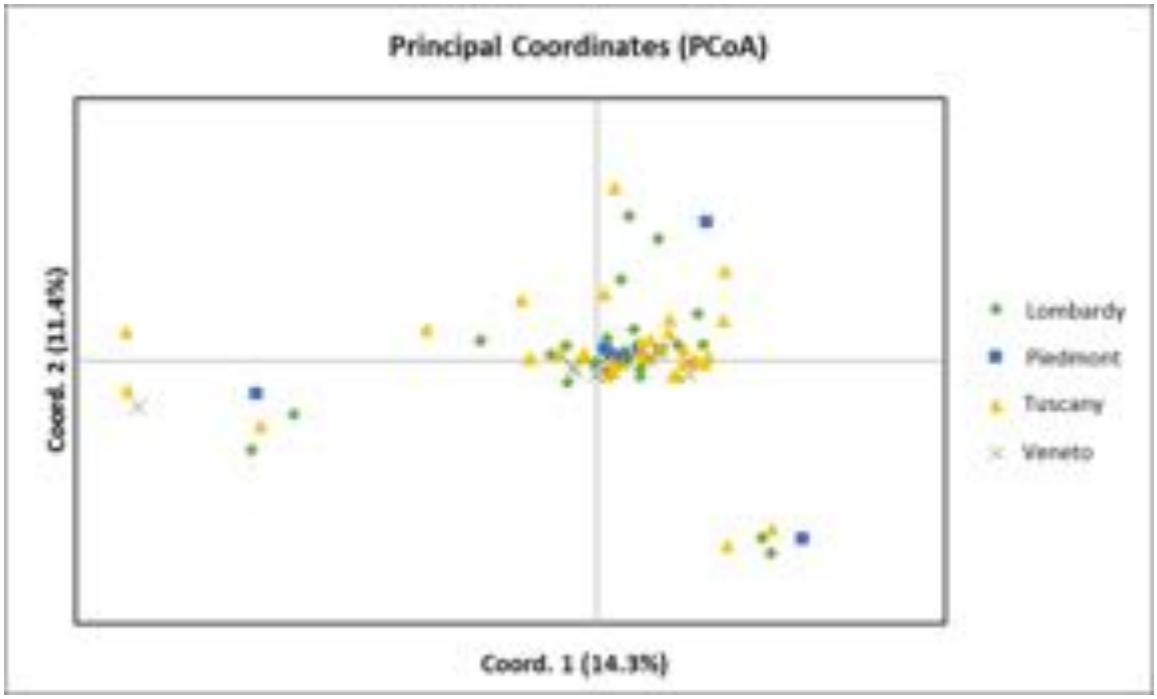
Nei's genetic identity showed a greater similarity between the groups of Tuscany and Piedmont (Table 4.21).

Table 4.21: Genetic differentiation measured by F_{ST} (above the diagonal) and *Nei*'s genetic identity (below the diagonal) between strains of *P. viticola* sampled in four Italian regions. *P* values for F_{ST} are in brackets.

	Lombardy	Piedmont	Tuscany	Veneto
Lombardy		0.000 (0.404)	0.003 (0.280)	0.011 (0.188)
Piedmont	0.986		0.000 (0.388)	0.000 (0.441)
Tuscany	0.987	0.991		0.010 (0.213)
Veneto	0.968	0.975	0.969	

The PCoA analysis confirmed the absence of a correlation between the genetic differentiation and the geographic origin, revealing a considerable overlap among genotypes belonging to the *P. viticola* strains clustered by regions (Figure 4.23).

Figure 4.23: PCoA plot conducted with Genalex 6.501 on pairwise genetic distances between *P. viticola* strains divided in four Italian regions.



Analysis on six clusters obtained by dendrogram

The PCoA analysis confirmed that the *P. viticola* strains did not group depending on the region of origin. A dendrogram was drawn to better understanding the structure of these populations (Figure 4.24).

The dendrogram was drawn using Mega4 under the rule of the Unweighted Pair Group Method with Arithmetic mean (UPGMA) clustering algorithm, taking into account the 96 *P. viticola* molecular fingerprints.

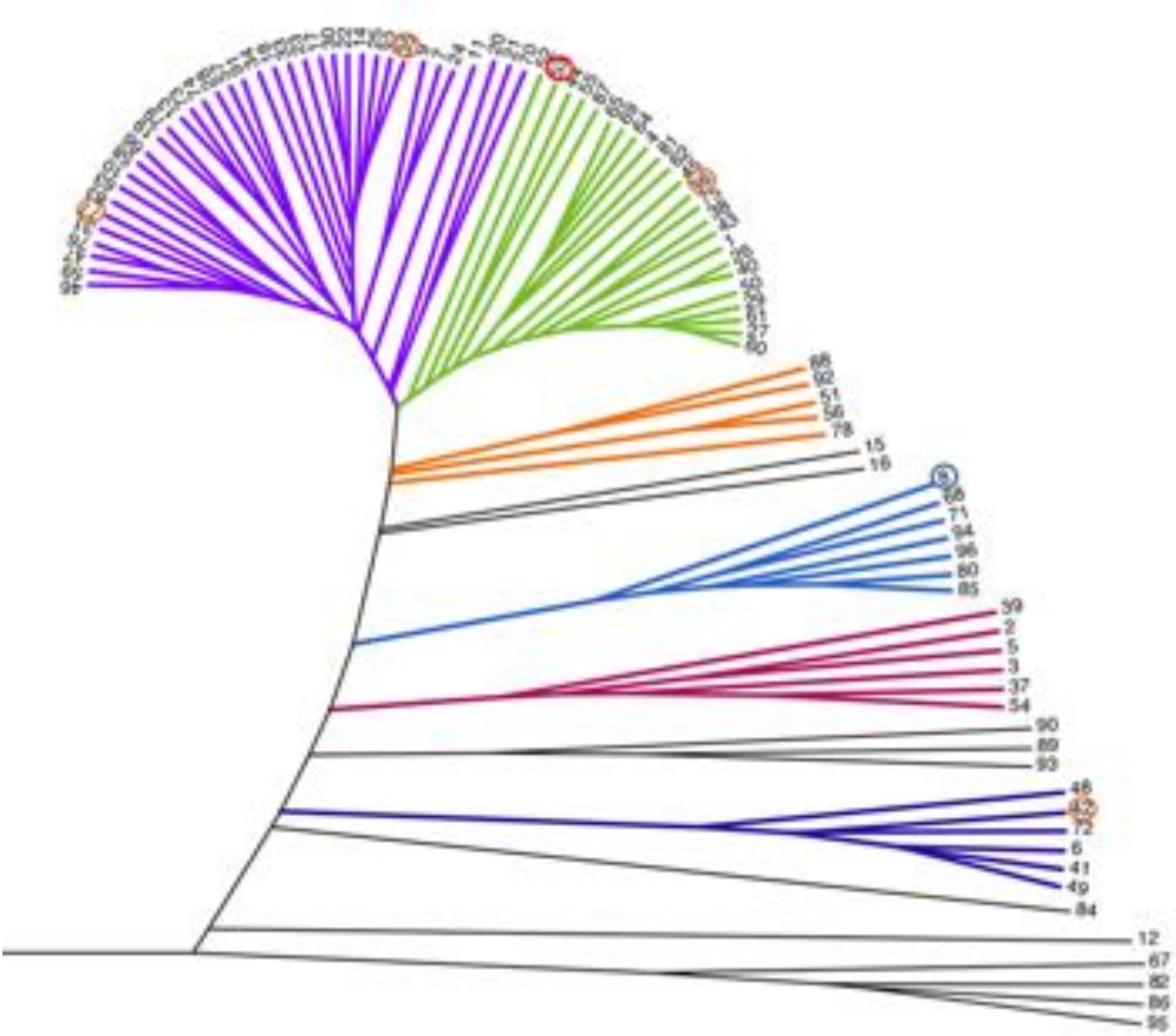
By the dendrogram six main clusters could be distinguished (expressed with different colours in Figure 4.24). The strains did not cluster according to the region of origin but two main subpopulation could be distinguished (the purple and green subpopulations); this two clusters seem to have a common ancestor (nodes) of those descendants.

Figure 4.24: UPGMA dendrogram of microsatellite obtained for *P. viticola* divided in 6 major clusters: cluster 1 (purple). cluster 2 (green). cluster 3 (orange). cluster 4 (light blue). cluster 5 (reddish-purple color). cluster 6 (blue).

High aggressiveness: red circles;

medium aggressiveness: orange

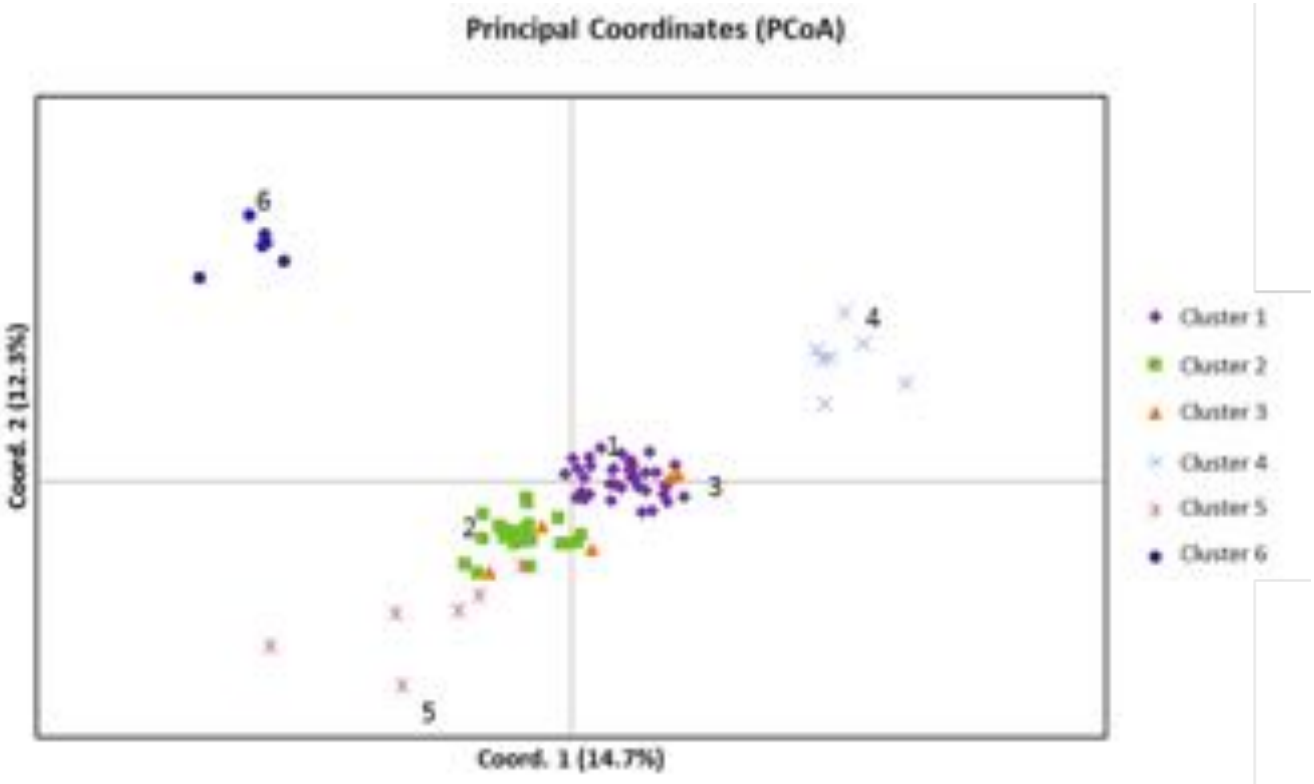
low aggressiveness: blue circles.



A PCoA analysis was conducted subdividing the *P. viticola* genotypes in the six clusters obtained by the dendrogram. The separation in groups obtained in the dendrogram was detectable also in the PCoA plot (Figure 4.25).

From the position of the strains evaluated for their aggressiveness in the dendrogram (Fig. 4.25), no relationship could be found between the level of aggressiveness and a particular cluster: in the same cluster can be found strains with high (in red), medium (in orange) and low (in blu) level of aggressiveness.

Figure 4.25: PCoA plot conducted with Genalex 6.501 on pairwise genetic distances from 6 *P. viticola* clusters obtained by the dendrogram.



In Table 4.22 are shown the three clusters, obtained by the dendrogram, characterized by private alleles. All the groups were characterized by at least one private allele, with the only exception of cluster 6. Cluster 1 was

characterized by a greater number of private alleles (5) followed by cluster 3 (4) and cluster 2 (2). As for the analysis conducted on the data set clustered by region, the frequency of each private allele was very low.

Table 4.22: Clusters obtained by the dendrogram with private alleles.

Cluster	Locus	Allele
Cluster 1	PV16	250
Cluster 1	PV148	126
Cluster 1	PV93	151
Cluster 1	PV139	135
Cluster 1	PV127	221
Cluster 2	PV31	241
Cluster 2	PV127	216
Cluster 3	PV31	237
Cluster 3	PV31	239
Cluster 3	PV16	245
Cluster 3	PV141	189
Cluster 4	PV101	262
Cluster 5	PV91	146

The AMOVA was calculated on the six clusters to evaluate the existence of significant genetic differentiations (F_{ST}) (Table 4.23).

The AMOVA showed that 54% of the total variation was distributed within clusters while 46% of the variation was explained by differences among groups with a total F_{ST} value of 0.26 (P=0.001) (Table 4.23).

Table 4.23: Analysis of molecular variance among and between clusters measured by F_{ST}.

Source	**df**	**SS**	**MS**	**Est. Var.**	**%**	F_{ST}	***P***
Among Pops	5	314.297	62.859	4.737	46%	0.26	0.001
Within Pops	79	445.080	5.634	5.634	54%		
Total	84	759.376		10.371	100%		

Significant genetic differentiation (F_{ST}) were detected among the *P. viticola* strains clustered by the dendrogram. The F_{ST} values were significant for all the comparisons. The highest *Nei*'s genetic identity and therefore the highest similarity was found for cluster 1 and cluster 3 while the lowest was found for cluster 4 and cluster 6 (Tab. 4.24).

Table 4.24: Genetic differentiation measured by F_{ST} (above the diagonal) and *Nei*'s genetic identity (below the diagonal) between the clusters of *P. viticola* clustered by the dendrogram. *P* values for F_{ST} are in brackets.

Cluster1	Cluster2	Cluster3	Cluster4	Cluster5	Cluster6	
-	0.124 (0.001)	0.085 (0.014)	0.293 (0.001)	0.304 (0.001)	0.396 (0.001)	Cluster1
0.930	-	0.101 (0.008)	0.373 (0.001)	0.301 (0.001)	0.410 (0.001)	Cluster2
0.945	0.936	-	0.311 (0.001)	0.224 (0.001)	0.399 (0.001)	Cluster3
0.918	0.851	0.886	-	0.490 (0.001)	0.556 (0.001)	Cluster4
0.921	0.927	0.875	0.824	-	0.479 (0.001)	Cluster5
0.848	0.846	0.806	0.753	0.804	-	Cluster6

Analysis on the two main clusters obtained by the dendrogram

The two main clusters obtained by the dendrogram analysis: cluster 1 and cluster 2 were more deeply investigated by AMOVA, in order to estimate the entity of the genetic differentiations (F_{ST}) (Table 4.26).

The AMOVA showed that 74% of the total variation was distributed within clusters and 26% of the variation was explained by differences among groups with a total F_{ST} value of 0.12 (P=0.001) (Table 4.25).

Table 4.25: Analysis of molecular variance among and between the two main clusters obtained by the dendrogram measured by F_{ST}.

Source	**df**	**SS**	**MS**	**%**	F_{ST}	***P***
Among Pops	1	59.405	59.405	26%	0.12	0.001
Within Pops	59	305.251	5.174	74%		
Total	60	364.656		100 %		

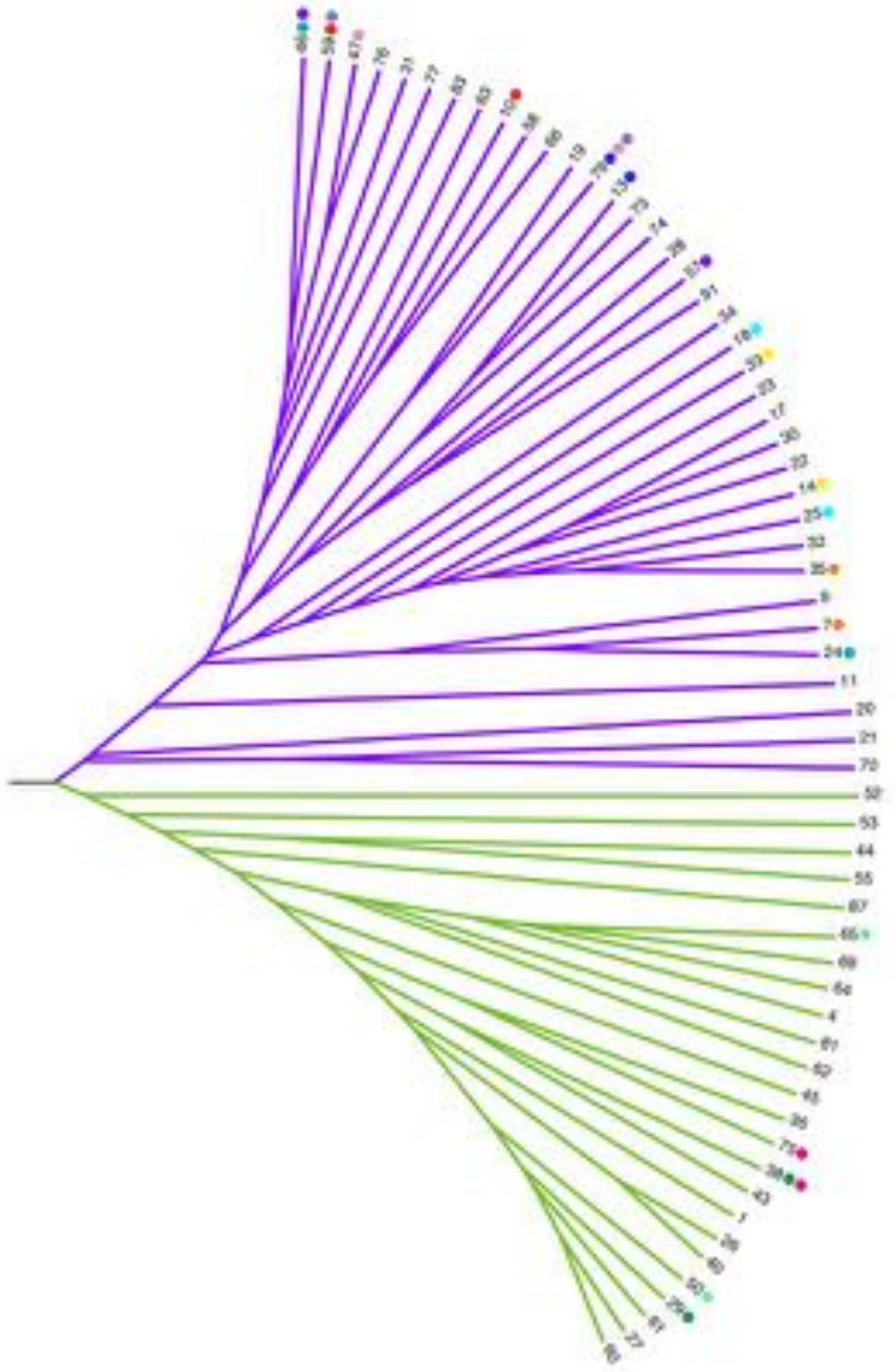
Coancestry analysis

Kinship analysis was carried out on the 96 *P. viticola* genotypes. For each pair of strains, the number of shared alleles to determine any possible parent-offspring relationship was estimated. Coancestry 1.0 software was used to estimate three relatedness coefficients: Δ_7, Δ_8 and r. These coefficients were calculated for each pair of genotypes. The values obtained using Coancestry 1.0 software were compared with theoretical values suggested in the Coancestry 1.0 User's manual.

$\Delta_7=0$
$\Delta_8=1$ } Parental-offspring
$r=1/2$

The most interesting result concern the parental-offspring relationship of cluster1 and cluster2: Cluster1 never showed parental-offspring relationship with cluster 2 and cluster 2 never showed parental-offspring relationship with cluster 1 (Figure 4.26).

Figure 4.26: Parental–offspring relationships of cluster 1 and cluster 2. The same coloured points indicate the kinship.



Structure analysis

A Bayesian clustering was performed on the 96 *P. viticola* genotypes with Structure 2.3.4 software varying *K* from 1 to 10. The highest *Ln P(D)* value was found for *K* equal to 5, indicating that the strains derived from 5 groups. For all the *P. viticola* genotypes, we inferred the proportion of ancestry in the four genetic clusters. All the genotypes had a membership coefficient equal to 0.2 in any of these clusters. These four groups found by Structure probably derived from one common ancestry (Fig. 4.27).

Figure 4.27: Bar plot graph of STRUCTURE results for *K* = 5**.** Each color represents one of the 5 inferred clusters (*K*). Individual lines are broken into color segments, with lengths proportional to the probability of each inferred cluster assignment.

5. DISCUSSION

P. viticola is the pathogen with the highest incidence on the reduction of production, causing several quantitative and qualitative drops on *V. vinifera* cultivars, traditionally used to obtain high quality wines.

Since the arrival of downy mildew agent in Europe in 1878, from North America, numerous attempts have been made to contain the pathogen through strategies based on cultural management and genetic improvement. The results obtained are not completely satisfying for the level of disease protection, in the first case, and for the quality of grapes obtained in the first breeding programs. At present, in areas with a high disease pressure the pathogen can be effectively controlled by chemical control. The European Directive 2009/128/EC attributes a great importance to the employment of integrated protection management (IPM) strategies and to unconventional protection strategies, highlighting the need of exploiting the sources of resistance present in the plants.

In this context, the Caucasian *V. vinifera* germplasm represents a source of great interest due to its high genetic variability, that could include characters of resistance towards pathogens such as *P. viticola*. This source of resistance, could be easily introduced in the cultivated *V. vinifera* varieties through simplified breeding programs, because the crosses would be made between members of the same species.

The evaluation of Georgian grapevine germplasm is interesting for different reasons. This germplasm comprises a very wide range of cultivars (525 according to Ketskhoveli *et al*., 1960) with high genetic variability (De Lorenzis *et al*., 2015) and various ampelographic characters, agronomical traits and phenological diversity (Maghradze *et*

al., 2012b). The viticultural and the enological features of this genetic material are also very different from Western European material (Imazio *et al*., 2013) and interesting because of their possible cultivation for innovative wine quality profiles, which could be different compared to wines from Western cultivars. There is also an interest in possible sources of useful genes for breeding programs for qualitative characters and/or for tolerance to biotic and abiotic stresses (Maghradze *et al*., 2012b, Quaglino *et al.,* 2016). Indeed, some interesting Georgian varieties showed a high level of resistance to *P. viticola* in experimental inoculations (Bitsadze *et al*., 2015).

In the present study, different approaches have been used to investigate the complex aspects of grapevine resistance to *P. viticola*. First, a screening activity was carried out on a collection of Caucasian accessions, wild and cultivated, to evaluate the possible presence of resistant characters towards *P. viticola.* Second, the inheritance of the resistance traits associated with resistance were investigated through the phenotypic characterization of the progeny obtained by crossing a resistant Georgian accessions, Mgaloblishvili, with varieties different susceptibility levels.

Third, a time-course observation of the Mgaloblishvili-*P. viticola* interaction at the confocal microscope was performed to clarify the quality and timing of the plant action on the pathogen.

It was proved that the host exerts selective pressure on the quantitative traits of the pathogen, and its ability to resist pathogens is important in this respect; once quantitative plant resistance is eroded, pathogens exhibited greater virulence not only on the resistant host, but also on fully susceptible hosts (Delmas, 2016). For this reason, pathogen features

involved in the durability of resistance were finally investigated through two different approaches: on the one hand it was investigated if the aggressiveness level of the pathogen could modulate the response of the resistant cultivar Mgalolishvili; on the other hand, the genetic structure of *P. viticola* belonging to different Italian regions was assessed.

The level of susceptibility of Caucasian varieties was investigated by combining bioassays with the evaluation of the disease incidence in field. Adopting this approach it was possible to obtain a more reliable evaluation of the plant behaviour and to get insights on the durability of resistance (Toffolatti *et al.,* 2016). *P. viticola,* in fact, has been shown to undergo differential adaptation to host cultivars, sometimes leading to erosion of partial resistance (Delmotte *et al.,* 2014).

The behaviour of the Caucasian accessions belonging to Georgia under field natural infection conditions was carried out on 94 Georgian Caucasian varieties cultivated in open field at the Regional Research Station of Riccagioia, in Torrazza Coste (PV), located in northern Italy. The disease incidence in field was assayed for three consecutive vegetative seasons. The downy mildew incidence was estimated also in an untreated plot of *V. vinifera* 'Croatina N', fully susceptible to *P. viticola*, located immediately nearby. A resistant control variety, *Vitis x labruscana,* was included in this analysis. Between 2014 and 2016, the disease incidence on 'Croatina N' ranged between 35 and 96 % on leaf and between 27 and 99 % on bunches. On the contrary, *V.x labruscana*, the resistant control variety, did not show any disease symptoms.

Even in presence of a high disease pressure, the Georgian accessions showed a very low disease incidence during the three years of analysis

recording 7.6% on leaves as highest value. Mgaloblishvili, the cultivar that showed good level of resistance in preliminary screening activity (Toffolatti *et al.,* 2016) underlined a resistant behaviour to natural infections, recording 1.69%, 1.59%, 0.2% on leaves.

On bunches the infection incidence was more variable. 'Kamuri Shavi N' showed the highest percentage of infected clusters, similar to that observed on the 'Croatina N' plot, during 2015 (86.5%). In 2014 and 2016 the number of infected bunches was very low. The season 2015 showed the major number of infected bunches with values comprises between 0 to 86.5%. In the Georgian plot, only five plants per variety were available, therefore the number of bunches were low, influencing negatively the results obtained in 2015. The Georgian accessions showed a reduced disease incidence also when the disease pressure was particularly high, as in 2014 and 2015.

Mgaloblishvili showed a low level of susceptibility showing low percentage of bunches with symptoms in 2014, 2015 and 2016, with values of respectively 0, 12.5 and 0.

The evaluation of downy mildew incidence in field can be influenced by different factors, suggesting the need of further investigations to confirm the behaviour of these Georgian accessions.

A huge collection of Caucasian and Iranian accessions were cultivated in pots at University of Milan screenhouse, located in Tavazzano con Villavesco (LO), in the province of Lodi. Experimental inoculations were carried out on leaves of these plants, to evaluate their behaviour towards *P. viticola.* In the screenhouse *V. vinifera* subsp. *vinifera* and *V. vinifera* subsp. *sylvestris* were cultivated. The Percentage Index of Infection (I%I)

were estimated for each plant. The 'Bianca' cultivar was added in each experimental inoculation, as resistant control, recording I%I equal to 0 or very low values. 'Pinot noir N' was used as susceptible control and registered I%I greater than 50% in all experimental inoculation assays. The screening analysis were repeated during the three consecutive years.

The analysis carried out on 148 Caucasian and Iranian cultivated varieties, showed that the accessions are, generally, characterized by high levels of susceptibility. The data exhibited a great variability, during the three years, in the capability of these plant to contain the disease. Only six accessions resulted resistant in two years (2014 and 2016): Dzvelshavi, Dondghlabi Shavi, Almura Tetri, Borchalo, Institutis Grdzelmtevana, Saperavi Khashmis.

The wild Caucasian accessions (*V. vinifera* subsp. *sylvestris*) showed a great variability in the pathogen response during the three years of analysis. In fact, in 2014 the disease severity data resulted widely distributed, assuming values comprises between 0 and 89.68% and 8 accessions resulted resistant. In 2015 no wild plants showed low levels of infection. On the contrary in 2016 most of wild accessions showed high level of resistance. However two accessions resulted resistant in both 2014 and 2016: Larchvali and Nakhiduri.

The inheritance of resistance genes, based on gene segregation mechanism, was investigated at the phenotypic level by analysing the progenies obtained by crosses of the Georgian cultivar Mgaloblishvili, that previously showed a good capability to contain downy mildew infections (Toffolatti *et al*., 2016).

The progenies obtained by the cross with Pinot noir resulted susceptible in both years of analysis. All individuals, in fact recorded percentage of infection very high.

Also most of the individuals originated by open pollination resulted susceptible, even if in the second year of experimental activities, more individuals presented an interesting behaviour. Only a single accession, the plant 7, presented a reduced I%I in both years.

The most interesting results were obtained by the analysis of the progenies derived from self pollination. The data showed a great distribution during all years. In 2014 the values were comprised between 0 to 99.9% and the 42.4% of the accessions resulted resistant. During 2015, more accessions resulted susceptible, and only 8.8% showed a resistant behaviour. During the last year, 39.6% of the individuals showed low I%I. Overall, only 2 accessions (124 and 147LIB) confirmed the resistant behaviour during the whole period of analysis.

These results get insight on the resistance mechanism: the progenies of Mgaloblishvili crossed with susceptible varieties (Pinot noir and other plants located in vineyard that had origin to individuals by open pollination) resulted susceptible. From this appears that, the genes involved in the resistant control were lost in the progenies, therefore, probably, the resistance genes are recessive. This hypothesis was supported by the results of the progenies obtained by self pollination. In fact, in this case, the individuals are characterized by a high level in the variability response towards *P. viticola* and a greater number of plants resulted resistant.

The variability in the plants response observed in the different years could be due to the different factors among which are: the physiological status of the plants; the aggressiveness level of the pathogen; and the influence of the environment. Since all the potted plants were kept in the same conditions in greenhouse and the resistant and susceptible controls always showed the same behaviour, we can keep the environment at a lower level of importance in this study. The same could be said for the physiological status of the plant, since the assays were carried out in spring on actively growing vegetation. In the present study, particular attention was paid to the *P. viticola* populations used in the experimental inoculations, because it was already observed that highly aggressive strains of *P. viticola* are able to create conspicuous damages, at the leaf tissue level, in resistant hybrids in comparison with against less aggressive strains (Toffolatti *et al*., 2012; 2016).

To evaluate the aggressiveness level of the pathogen and how it can modulate the response of Mgaloblishvili, different *P. viticola* strains were inoculated at the same time. Bianca and Pinot noir were used as resistant and susceptible controls. Investigating the host response to individual *P. viticola* strains provides not only a profile of resistance but also insights on the evolutionary potential of the pathogen strains through the investigation of their aggressiveness, defined as degree of damage caused to the host, and their fitness *i.e.*their ability of surviving and reproducing (Toffolatti *et al.,*2012). After 7 days after inoculations, when the sporulation occurred, the sporangia were collected from each plants samples, in order to count the number of differentiated sporangia, a component of the pathogen fitness.

The quantitative assessment of the disease intensity showed that the

pathogens strains possess different aggressiveness levels. Pinot noir showed different classes of infection: the values were comprised between 26.2% and 90.5%. The behaviour of Mgaloblishvili was very variable. In presence of pathogens characterized by high aggressiveness level, Mgaloblishvili showed a behaviour similar to that of Pinot noir. On the contrary, in presence of medium-to-low aggressive pathogens, it behaved like the resistant accession Bianca. The most virulent strain, without any reduction in its overall fitness, induced the same disease intensity in Pinot noir and Mgaloblishvili, suggesting that no fitness costs are associated with the resistance breakdown (Toffolatti *et al.*,, 2012). These results were confirmed by the analysis on the number of sporangia produced by the pathogen, which were strictly related to the infection degrees.

The host-pathogen interaction has a considerable role to define the entity of disease and, in consequence, the behaviour of the host. Resistance to *P. viticola* involves a complex mechanism, composed by genetic factors, whose expression is induced by the pathogen, and preformed physical barriers of the plant. Among these, are leaf hairs, that could constitute a physical barrier to the pathogen penetration in the stomata (Kortekamp and Zyp rian 1999). However, at least in the accessions investigated, the leaf hair intensity could not be related with resistance to *P. viticola* (Toffolatti *et al.*, 2016) Among the induced physical barriers, callose has a role in limiting the pathogen growth in the host (Kortekamp *et al.,* 1997).

Histological analysis on Mgaloblishvili, aimed at investigating the pathogen development in the host tissues, the apposition of callose by the host and the timing of the plant response. Bianca and Pinot noir, were used as resistant and susceptible controls. Also in this case, the leaf tissues

were inoculated at the same time and kept at the same conditions.

Several differences were observed between Pinot noir, Bianca and Mgaloblishvili. In Bianca, the resistant variety, the defence response was early activated, since HR reactions was detected already 24 hours after inoculation, as demonstrated by the absence of mycelium and the great quantity of callose apposition synthesized in the proximity of the stomata, constituting a physical barrier to penetration and evasion of sporangiophores, and explaining the absence of sporulation observed in experimental inoculations.

On the contrary in the susceptible cultivar, Pinot noir, a regular development of the pathogen occurred: the hyphae extended from the substomatal vesicle in the leaf tissues, forming regularly shaped haustoria. Six days after inoculation the leaf tissue was completely invaded by the pathogen, that produced sporangiophores bearing sporangia from single stomata..

Mgaloblishvili showed a different behaviour from that of both Pinot noir and Bianca. In fact after at 48 hours after inoculation there were no differences between Mgaloblishvili and Pinot noir, whereas Bianca had already blocked the pathogen. 72 hours after inoculation, Mgaloblishvili clearly differed from Pinot noir in relation to the pathogen structures. *P. viticola* mycelium in Mgaloblishvili appeared not defined, characterized by hyper-branched hyphae with an excessive number haustoria, indicating a deregulation of the pathogen growth. Six days after inoculation, in Mgaloblishvili degenerated mycelium clearly appeared and callose appositions were visible. The callose appositions located into the mycelium, suggesting that they were synthesized by the pathogen,

probably, in order to delimited the dead parts of the mycelium. Numerous sterile sporangiophores, hyperbranched, emerged from the stomata. The extensive leaf tissue areas, colonized by the pathogen, and the differentiation of sporangiophores, could to clarify the level of infection observed in the experimental inoculations. Probably the sporulation occurred, but the plant activated the defence response.

The genetic structure of different *P. viticola* strains belonging to different regions were analysed in order to investigate the genetic diversity of the pathogen populations in Italy. The analysis was carried out using 21 microsatellite loci developed by Rouxel *et al.*,(2012), Delmotte *et al.* (2006) and Gobbin *et al.* (2003) on 96 *P. viticola* strains. Among all the strains, no clone was found.

Concerning the expected heterozygosity (H_E), six loci (PV31, PV16, PV65, PV104, PV142 and PV127) displayed strong heterozygote deficiency compared to what would be expected under Hardy–Weinberg equilibrium ($P < 0.001$). Four of these loci (PV65, PV104, PV142 and PV127) displayed significant, high levels of heterozygote deficiency and had much higher proportions of missing data. However, these markers could be affected by the high presence of null alleles. Therefore we can conclude that the Italian *P. viticola* population is predominantly panmictic as reported by other Authors (Gobbin *et al.*, 2003; Fontaine *et al.*, 2013).

The average value of H_E get insights on the genetic diversity of a population. In the present study, the average value of heterozygosity (0.32) is analogous to that found by Fontaine and coworkers (2013), confirming that *P. viticola* possess a lower value compared to other invasive oomycete species (Fontaine *et al.,* 2013).

First the genotypes were clustered on the basis of the region of origin in order to elucidate if geography could have a role in shaping the genotypic variability of the *P. viticola* strains. The data set was divided in four populations based on the Region of origin: Lombardy, Tuscany, Piedmont and Veneto. The PCoA and AMOVA showed that there were no differences among populations (0% of the observed variance came from the variation among each population) and, furthermore, there was the absence of structure in relation to the geographic origin of the strains. The index of fixation (F_{ST}) and the genetic distance (*N*ei) confirmed the hypothesis of the absence of a geographic differentiation among all the populations. By the results of these analyses, we can conclude that in Italy the genetic differences were not related to geographic factors.

The presence of groups in the entire dataset of *P. viticola* genotypes was investigated by the analysis carried out with Mega4. In the dendrogram two main clusters and four smaller clusters were evident, all descendants from a single branch. The following analyses were performed on the data set clustered in six groups, taking into account the subdivision observed in the dendrogram. This subdivision was confirmed also by PCoA analysis. The two bigger groups, cluster 1 and 2, appeared very close in the dendrogram and in the PCoA plot, but only small overlaps were revealed. Based on AMOVA, even if the most of the genetic diversity was observed within population, the percentage of variation among populations resulted very high (46%).

To evaluate the entity of the genetic difference between the two main clusters found by the dendrogram, different analysis on the genotypes of these two clusters were performed. The AMOVA revealed a great genetic diversity between the two groups, with a percentage of observed variance

among them of 26%. With the support of the COANCESTRY software it was possible to analyse the parent-offspring relationship between these two clusters. This kind of kinship was found only within each clusters but never among the two clusters. The fixation index (F_{ST}) confirmed the presence of a significant differentiation between the two groups.

Finally, a Structure analysis was performed on the entire dataset without giving any information on the subdivision of the genotypes into groups. The Bayesian clustering showed that the best assignment was found for K=5, indicating that the strains derived from 5 different clusters. The proportion of the membership coefficients of each genotype to each of the five clusters was equal and amounting to about 0.2: this equal proportion of all the genotypes to the five classes indicates that all the *P. viticola* genotypes derive from the same ancestor. Probably the absence of parent-offspring relationship among these clusters may reflect the increased genetic difference. These two clusters are differentiated from the same branch. We can hypothesize that all the genotypes investigated in the present study derive from a unique ancestor, probably deriving from the same population arrived in Europe from North America. (Fontaine *et al.,* 2013).

This theory is in line with the hypothesis formulated by Fontaine *et al.* (2013), stating that in Europe there are two weakly differentiated genetic clusters of *P. viticola*. Fontaine supposed three different theories about the development of the two clusters: the first assumes that *P. viticola* was first introduced into Western Europe from an unsampled source population, from which it was subsequently introduced into Eastern Europe; the second theory assumes the reverse case; the third assumes that the two groups were introduced into Europe independently, but from the same

source population (Fontaine *et al.,* 2013).

In order to evaluate if the genetic differences could modulate the aggressiveness level of the pathogen, the strains used for the experimental inoculations with single strains were included in the genetic analysis. The strains with a high or low level of aggressiveness resulted admixed in the two main clusters found in the dendrogram. Therefore, it can be excluded that the aggressiveness level is a factor influencing the genetic differences. It could be interesting to investigate if the genetic differences are related to other specific pathogen characteristics, increasing the number of samples.

In conclusion, the Georgian *V. vinifera* subsp. *vinifera* and *sylvestris* accessions screened for resistance to *P. viticola* in the present study denoted, in general, a good resistance level, expecially in field assays. The experimental inoculations carried out on Mgaloblishvili, the Georgian resistant variety, and its progenies indicate that the resistance character is probably recessive, since only the progenies deriving from self pollination kept the resistant phenotype. Experimental inoculations with single strains and histological analysis showed that Mgaloblishvili activates a defence response towards *P. viticola* that was visible 48 hours after inoculation, but the host response was modulated by the aggressiveness level of the pathogen.

In this study, the first one aiming to extensively analyze the genetic structure of *P. viticola* populations in Italy, genotypic differentiation was not detected in strains belonging to different regions, but was evident in subgroups, which did not differ for the aggressiveness level. This result is completely in line with the theory formulated by Fontaine and coworkers

(2013).

The results obtained in the present study contributed to the development of a RNAseq project that aims at elucidating the genes expressed by Mgaloblishvili in response to the pathogen and to the QTL characterization that is currently under investigation at Fondazione Edmund Mach.

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