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Study of *Venturia inaequalis* sensitivity to fungicides through molecular and biological methodologies

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Esame finale anno 2013

DEDICATION

This dissertation is dedicated to my parents and brother, for their sacrifice, unconditional love, support and being there for me always. I love you.

“Peace at home, peace in the world”
Mustafa Kemal ATATURK

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1. Summary

Apple scab caused by *Venturia inaequalis* (Cke.) Wint. is one of the diseases with the highest economic importance both in Italy and in Turkey as in other countries and requires a high number of fungicide applications. The introduction, in the late 1990s, of strobilurin fungicides led to an improvement in disease control. These fungicides specifically inhibit cell respiration by binding at the ubiquinol oxidation centre (Qo site) of the mitochondrial cytochrome bc1 complex. After a few years of excellent protection, several cases of reduced scab control caused by pathogen resistance to strobilurins (QoI) were reported in many countries. Resistance is caused by a mutation which results in a change of Glycine to Alanine at position 143 of cytochrome b (G143A substitution).

This study was aimed to correlate the results of relative germination from *in vitro* tests with those of qPCR on sixty-four *V. inaequalis* populations and sixty monoconidial isolates. Samples were collected in Italian and Turkish distinct locations from orchards with different scab management.

In vitro assays were carried out in petri dishes and concerned the conidial germination for the populations and monoconidial isolates. Also, the mycelial growth was evaluated for the monoconidial isolates. Trifloxystrobin was used as active material to achieve the following concentrations: 0.0001, 0.001, 0.01, 2 mg L^{-1}. According to test results EC_{50} values and relative germination (RG) were calculated.

In molecular analysis, genomic fungal DNA of populations was extracted directly from the infected leaves by a CTAB-based method. In order to obtain the genomic fungal DNA of monoconidial isolates, spores were used as extraction material utilizing Chelex 100 chelating resin. CAPS analysis were performed on all samples by two specific primers ANK 10 and ANK 283 to amplify a 413 bp fragment of cytochrome b of *V. inaequalis* and this fragment include the G143A position. Then PCR products were digested with the Tse 1 restriction enzyme which is able to recognise the mutation causing G143A substition. In this

study, an allele-specific qPCR with primer sets designed was successfully developed to determine the frequency of QoI-resistant allele (A143) by SybrGreen. The forward primers FwS5418 and R5548 were used respectively for sensitive and resistant allele, while reverse common primer, R5548, was used. With the aim to verify the qPCR efficiency (E) and specificity of each primer pair, different percentage of referent sensitive and resistant plasmidial DNA were analyzed. The ratio of the two alleles presence in samples was calculated using ΔC_q method.

The correlation between the results of two assessment methods showed that quantitative assessments using qPCR followed a similar pattern to that obtained using *in vitro* conidial germination test in very sensitive and very resistant populations. In fact, in most cases, it was observed when RG was < 10%, mutated allele frequency was < 10% and when RG was > 70%, very high mutated allele (>80%) was detected. Some variability between two test results was observed in hetereogenous populations. Therefore, the results of correlations between *in vitro* and qPCR showed a positive but not very high correlation for *Venturia inaequalis* populations (R^2=0.70). On the contrary, this correlation between two assessment methods was very high for monoconidial isolates (R^2=0.92). qPCR assessment was highly representative of the results obtained by *in vitro* assay for monoconial isolates.

The method developed here was designed as alternative to traditional methods and qualitative tests and showed a better sensitivity than the CAPS method and *in vitro* tests. However, not a very high correlation between biological and molecular data was observed in hetereogenous populations. It was observed that using qPCR method makes it possible to measure the mutation level in DNA isolated from viable and non-viable fungal material. Therefore, results obtained in quantitative PCR and from traditional spore germination assay differed for the same fungal population and in some cases it is difficult to assess the resistance in the field by only qPCR. Moreover, the mutation does not always explain the QoI-resistant phenotype.

qPCR allows a rapid detection of mutation with known resistance mechanisms, at low frequencies, and even if it is not possible to have a precise correlation between biological and molecular data, it is possible to observe that populations classified as sensitive *in vitro* tests have generally a rather precise range of percentage of mutated alleles, and the same can be observed on resistant populations where the percentage of mutated alleles is generally superior to certain values. However, it is not always possible to correlate the frequency of detection of the mutation with biological assessment.

In conclusion, in such situations monitoring by molecular techniques must be supported by standard *in-vitro* resistance assessments and observation of field performance in order to have a more reliable conclusion on sensitivity of each *V. inaequalis* population to strobilurins.

2. Introduction

Apple scab, caused by the ascomycete *Venturia inaequalis* (Cke.) Wint, is the most important disease of apple (Malus x domestica Borkh.) worldwide in terms of economic cost of control. However, the disease is more severe in cool, moist climates during early spring (MacHardy, 1996; Manktelow *et al*., 1996). It is not known when scab first appeared in orchards. The first report on scab was published by Fries in Sweden in 1819 (Fries, 1819). *V. inaequalis* has a wide geographical range and is found in almost all areas in which apples are grown commercially. The disease can affect 70 % or more of the yield if control measures are not applied well (Agrios, 2005).

Taxonomy of apple scab is as follows;

Teleomorph: *Venturia inaequalis* Cooke (Wint.)

Kingdom: Fungi

Phylum: Ascomycota

Subphylum: Euascomycota

Class: Dothideomycetes

Family: Venturiaceae

Genus: *Venturia*

Species: *inaequalis*

Anamorph: *Fusicladium pomi* (Fr.) Lind or *Spilocaea pomi* (Fr.) (Bowen *et al*., 2011)

2.1 Biology and life cycle of apple scab

Venturia inaequalis Cooke (Wint) is a hemibiotrophic fungus and has two phases characterized by a saprophytic phase or the perfect state (sexual) in autumn-winter and a parasitic phase or the imperfect state (asexual) in spring-summer.

V. inaequalis survives the winter primarily in diseased apple leaves on the ground as immature pseudothecia (fruting body) (Fig1) but also the fungus can survive in infected bud scales and on twigs, as mycelium or conidia on some cultivars and in certain regions (Holb *et al.,* 2005). Pseudothecium is seen as black pin-point heads on the over-wintering apple leaves. Pseudothecia and ascospores develop in these leaves. In spring meiosis and mitosis take place and the diploid pseudothecium is forming numerous asci containing 8 ascospores (12-15 x 6-8 µm) each (Sivanesan and Waller, 1974). Each pseudothecium contains 50 to 100 asci (Agrios, 2005). Ascospores are the primary source of inoculums and infection is initiated in spring and early summer. Apple scab ascosopores are the one-septate green, yellowish, or olivaceous-brown (Barr, 1968) and consist of two cells of unequal size (Agrios, 2005) (Fig 2). These ascospores are discharged in rain, and leaf wetness and they are carried by the wind to the developing fruit and shoot buds where they cause primary infections that can lead to lesions and production of conidia. Most spores (90-95%) are discharged during daylight between the pink bud to petal fall stages. The 5-10% of ascospores discharged at night is enough to start an epidemic (Rosenberger, 2012).

In spring, only the youngest 2-3 leaves are susceptible to infection. Early in the season, scab spots first appear on the underside, later in the season, lesions are more likely to appear on the upper surface of leaves.

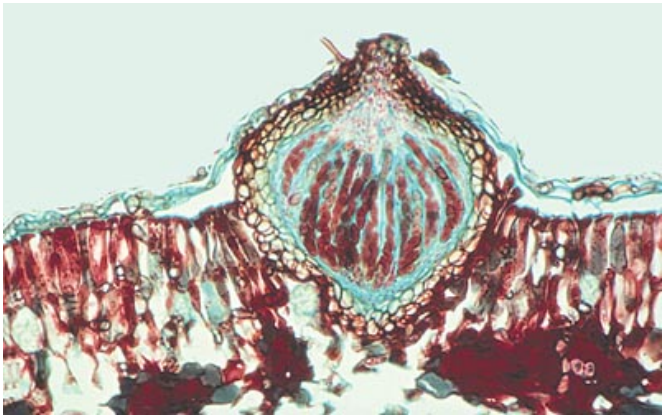


Fig.1. Pseudothecium of *Venturia inaequalis* containing asci and ascospores (http://www.apsnet.org/edcenter/illglossary/Article%20Images/Forms/DispForm.aspx?ID=643)

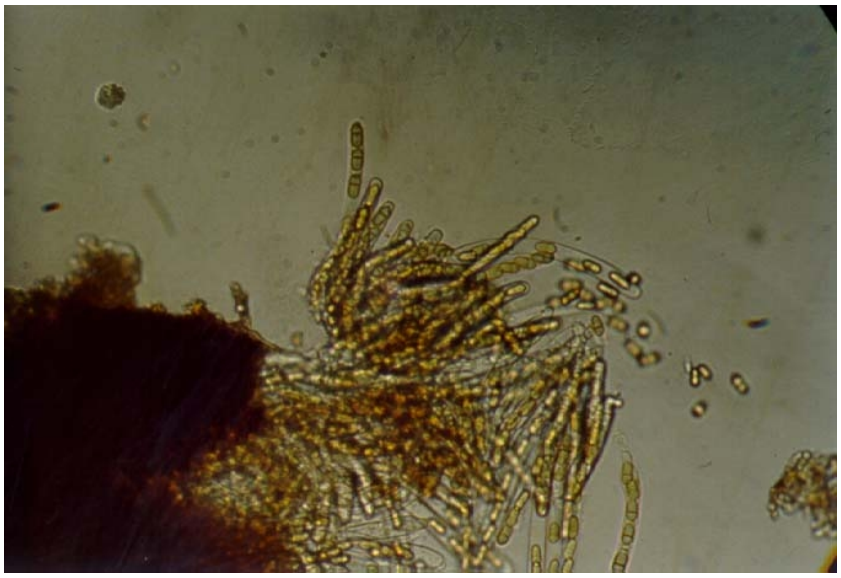


Fig. 2. Ascospores of *Venturia inaequalis* in asci (Brunelli)

The length of time required for ascosopore's germination and penetration on leaves, or fruits depends on the number of hours of continuous wetness and the temperature during the wet period. The most famous and complete relationship published by Mills and Laplantec (1951) (Fig 3).

During germination, ascosopore germination tube goes into the cuticle of leaf or fruit and grows between outer cell wall of epidermis and the cuticle. A fungal mycelium forms here and pushes up through the leaf cuticle and ruptures it. The mycelium of *V. inaequalis* is

septate, and the nuclei are haploid Mycelium is the mass of conidia and conidiophores and they cause the olive-green, velvety scab lesions (MacHardy, 1996).

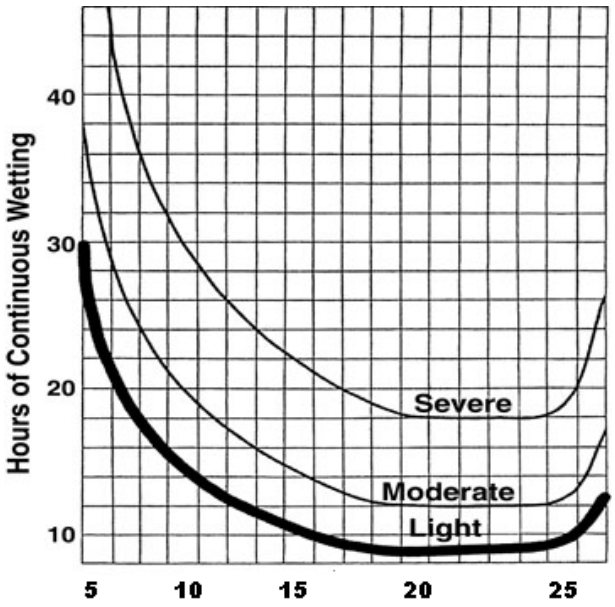


Fig. 3. Relationship of temperature and duration of leaf wetness to the severity of apple scab (Agrios, 2005).

The conidia are single-celled, uninucleate and measure between 6 and 12 µm wide and between 12 and 22 µm long (Fig 4). Infection is highly influenced by the susceptibility of the cultivar, weather conditions and the quantity of conidia. Up to 100,000 conidia can be produced by a single lesion (Vaillancourt and Hartman, 2005).

In summer, conidia (asexual spores) can be carried throughout the orchard onto other leaves and fruit within the tree by water or wind, and cause numerous new, secondary infections. These infections produce more conidia and continue the disease cycle. Secondary infections continue during cool, wet periods of spring, early summer and autumn until the leaves and fruit fall from the tree.

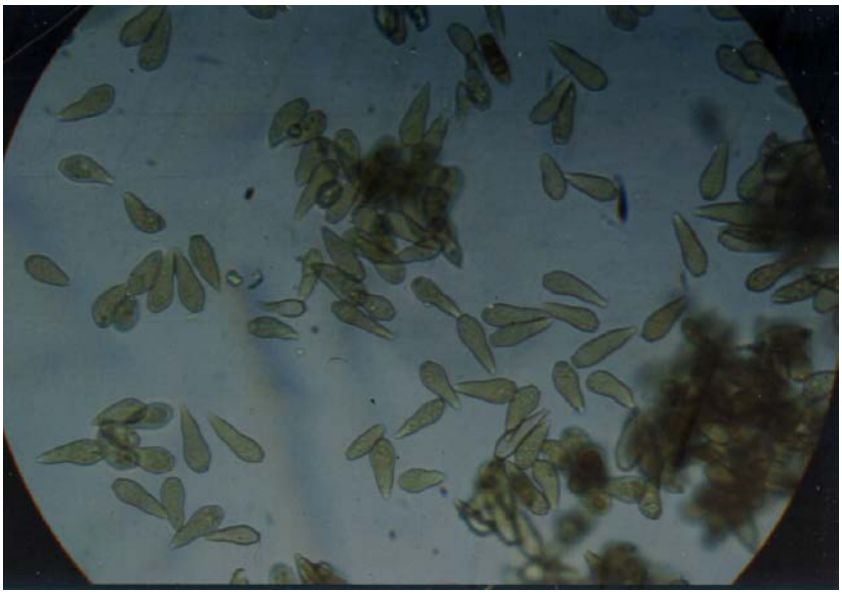


Fig. 4. Conidia of *V.inaequalis* (Brunelli)

V. inaequalis go through sexual reproduction on fallen infected leaves over winter and pseudothecia are formed. They will mature during winter and develop a new generation of ascospores for the following spring to start a new cycle (Fig 5).

2.2 Symptoms of apple scab

The Apple Scab fungus does not kill the tree, but infection results in leaf and fruit loss. Apple scab infects leaves, fruit, petioles, blossoms, flowers, sepals, pedicels, young shoots, and budscales. The symptoms are generally most noticeable and serious on leaves and fruit.

The first visible lesions on the leaves are often found on the lower surfaces of leaves in the spring and they are small, discrete, and olive to greenish-black. The spots can be seen on upper side later in the season. Symptoms on the upper surface are more distinguished (Fig 6). Initially, infections appear as olive-green spots with indefinite borders. In time, spots become larger and velvety olive-green collared by the numerous conidia. Olive green lesions turn gray brown with distinct margin and lesions are raised.

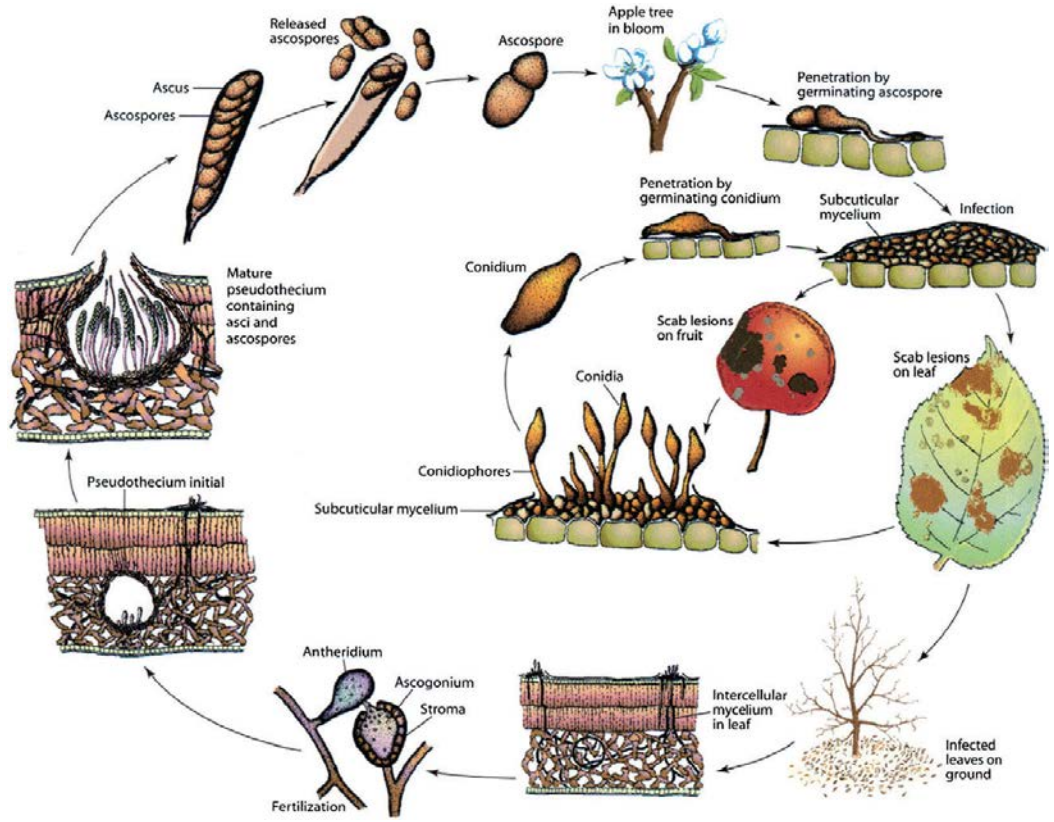


Fig. 5. The life cycle of *Venturia inaequalis* (This diagram was published in Agrios, *Plant Pathology*, p. 506. Copyright Elsevier2005.)

The lesions on the fruits are similar to those on the leaves. A young fruit infections result in deformation and cracking. Spots on the apple fruits are superficial, darker-colored and enlarge more slowly than on leaves (Fig 6). Lesions become brown, corky and scabby. Fruit infections that occur in late summer or early fall may not be visible until the fruit are in storage (Pin-point scab lesion) (MacHardy, 1996).

Fig. 6. Scab symptoms on the upper surface of leave and on the fruit (Brunelli)

2.3 Fungicides used to control apple scab

Apple scab management is based on fungicide applications. Fungicide spray programmes are required in most apple-growing regions during spring and summer. A variety of fungicides with differing modes of action are available. Scab is currently controlled by up to 15–20 applications of protective and curative fungicides during the growing season (Jamar *et al*, 2007).

Apple scab first became an important problem in Europe in the 1880's. Bordeaux mixture was the main chemical control of scab before 1920 (MacHardy, 1996). Then sulphur-based products were started to use and became popular in the mid-1930's (MacHardy, 1996) together with other inorganic fungicides (copper, mercury). Due to some of negative properties of sulphur and copper materials (injuries to the foliage and fruit, difficulty of preparing, required numerous applications, etc.), the new organic fungicides entered in the market. The origin of fully synthetic fungicides can be dated to 1950s when the dithiocarbamates were discovered. Dithiocarbamates as protectant fungicides were the first organic fungicides that controlled apple scab, especially mancozeb. Chlorothalonil was introduced in 1963-64 and widely used for apple scab control (Morton and Staub, 2008). In

1960s, dodine was entered to market as an effective protectant and eradicant fungicide. Dodine was used as the first local systemic organic fungicide. Although dodine can be active when applied in pre- and postsymptom, its efficacy is weak in after-infection applications for scab control (Köller *et al*, 1999). Dithianon was an another important protectant fungicide in 1960s against apple scab.

From the nineteen-sixties, synthetic fungicides with several new modes of action came on the market. In the late 1960s and 1970s, more new fungicides with novel structure and mostly with systemic activity like benzimidazoles were introduced. Since their introduction, systemic fungicides have gradually replaced the older non-systemic products. During these years, research and development enlarged rapidly along with growth of the fungicide markets and many of the fungicides were registered after introduction of benomyl. The single-site inhibitor benomyl was introduced to the market as the first systemic fungicide in 1967. The type of activity of fungicides allowed establishing different treatment application: protection, after infection, presymptom and postsymptom (Szkolnik, 1978).

Apple scab since 1980 has been controlled by systemic sterol biosynthesis inhibitor fungicides (DMIs), anilinopyrimidines, strobilurins and guanidines. DMIs represent one of the largest groups of systemic fungicides that have been used to control fungal pathogens (Zhan *et al*., 2006) and DMIs are most active for apple scab in an after-infection mode of application (Köller *et al.*, 1999). In the beginning of 90s a new class of fungicides, strobilurins became important to be effective, protective and eradicant.

These systemic fungicides all have very specific modes of action that allow for the development of resistance.

2.4 Fungicide resistance in *Venturia inaequalis*

With the introduction and widespread use of single-site fungicides, the evolution of resistant strains of pathogens has been rapid and fungicide resistance has become an increasingly serious worldwide problem in the agrochemical industry. During the past years, important problems of fungicide resistance development have been reported. Resistance is a stable and inheritable adjustment by a fungus that results in reduced sensitivity to a fungicide. This means, that fungicides don't affect or less affect on resistant isolates and they can become dominant in local pathogen populations under the selection pressure of fungicide use. The resistant isolates can reproduce and spread to large areas and this can cause epidemics and economic losses.

All fungicides with a single-site mode of action (MOA) have an high potential to cause fungal resistance. Many types of resistance mechanism are known and the most common resistance mechanism is an alteration of amino acids of the biochemical target site of the fungicides.

Fungicide resistance in *V. inaequalis* is first occured for dodine in the late 1960s (Gilpatrick, 1982) and dodine resistance became widespread during the 1970s (Köller *et al*, 1999). *V. inaequalis* has successively developed resistance to benzimidazole (benomyl) and resulted in specific mutations in the gene encoding the target protein β-tubulin that modify amino acid 198 (from glutamate to alanine, lysine or glycine) (Koenraadt *et al*., 1992). Resistance has already been acquired to anilinopyrimidines (Küng *et al*., 1999) and the mode of action includes inhibition of methionine biosynthesis and secretion of hydrolytic enzymes.

There is also increasing evidence of DMI and strobilurins. *V.inaequalis* sensitivity reduction to demethylation inhibitors (DMIs) has been documented by Sholberg and Haag in 1993 and Köller *et al*., in 1997. The mechanism of resistance to the DMI fungicides is

known the overexpression of the target-site CYP51A1 gene (14a-demethylase gene) from *V.inaequalis* (efflux mechanism) (Schnabel and Jones, 2000).

Not long after the introduction of strobilurins in the market, strobilurin resistant isolates of *V.inaequalis* were detected for the first time in 1997 in European field (Fontaine *et. al*, 2008) (More details are in mechanisms of resistance to QoI fungicides section).

2.5 Fungicides resistance in *Venturia inaequalis* in Italy and Turkey

A key role in apple scab control has traditionally been played by mobile products such as dodine, benzimidazoles and DMIs, also thanks to their curative activity. Since the late nineties, further improvements have been possible thanks to new anilinopyrimidines and strobilurins that respectively assured a good curative activity in both country and made it possible to extend spray intervals even up to ten days between treatments (Delen, 2008; Brunelli *et al.*, 2002).

Italy

Apple scab is the main disease of apple in Italy and requires numerous fungicide applications, especially in northern areas because of frequent rains and fairly high temperatures in first growth stages. In the early seventies, the key products, benzimidazoles were dramatically involved in resistance development after few years of introduction to the market. This led to a return to the use of dodine until the late seventies, when fenarimol started the SBI period. These fungicides predominated for many years with triazoles (bitertanol, myclobutanil, penconazole, etc.) but were progressively affected by resistance in the middle of the 80s (Fiaccadori *et al*., 1987). After some years of excellent performances, apple scab control difficulties have been reported since 2000 and 2001 in Northern areas of Italy with programmes based on strobilurins (Table 1) and/or anilinopyrimidines (Fiaccadori *et al*, 2005). The field control complaints have in the last few years in Italy led to reconsider

the use of these fungicides that in the late nineties became the key products against apple scab. Strobilurins were abandoned in some areas, especially where control failures were rather frequent, or utilized only in low risk periods, while in others areas they continue to be applied with usual antiresistance recommendations. On the contrary, anilinopyrimidines continue to be used in most Italian apple areas, often mixed with products characterized by other mechanisms of action.

In the last few years, the reduction of effectiveness reported in some areas for strobilurins and anilinopyrimidines led to a strong increase of the use of other fungicides in *V. inaequalis* control in Italy, mainly dithianon among contact products and difenoconazole among systemic ones. Complaints of reduced control have recently been reported also for these fungicides and a survey was started. The first results obtained by *in vitro* sensitivity tests of *V. inaequalis* to dithianon did not confirm the suspicion of resistance, leading to the conclusion that control complaints could be caused by an unsuitable timing of applications of this fungicide in high infective periods (Fiaccadori *et al*., 2012).

Table 1. Authorized strobilurins on apple scab in Italy

Strobilurins	Dose	Formulation	Authorization Date
kresoxim-methyl	10-14g/100L	WG	27.03.1997
trifloxystrobin	10-15g/100L	WG	06.12.2002
pyraclostrobin (12.8%) +boscalid (25.2%)	55g/100L	WG	13.07.2006

Turkey

Apples can be grown in almost all parts of the country and fungicides are widely used to control scab disease. In Central Anatolian Region, especially in Egirdir (Isparta) where apple production is high and a lot of fungicides are applied (Fig 7)**.**

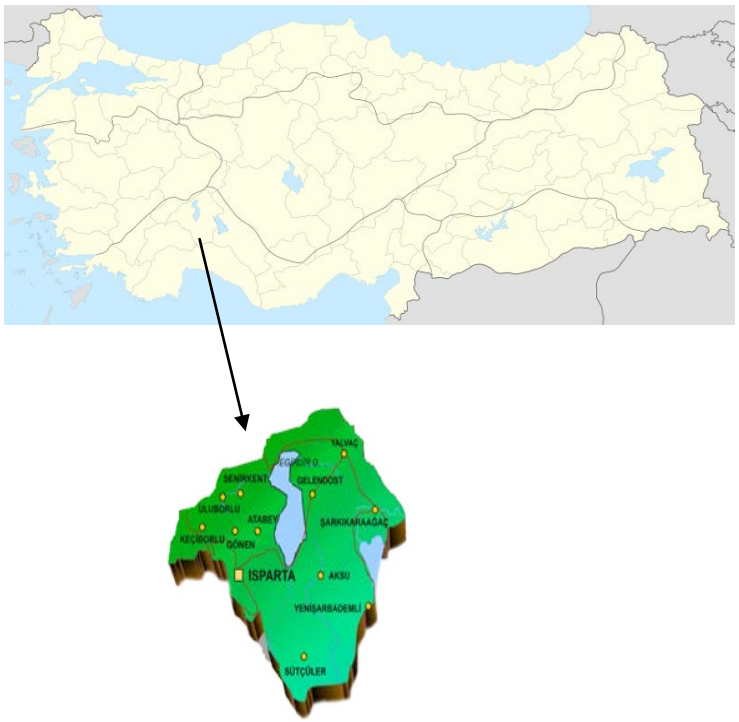


Fig.7. Map of Isparta region in Turkey

Failure to control disease has been observed in some orchards and fungicide resistance was claimed to be the main reason. There are some unpublished studies from the chemical industry testing the sensitivity of the pathogen's local isolates to commonly used fungicides. Fungicide resistance in *V. inaequalis* is well known in Turkey for old fungicides such as dodine, benzimidazole, demethylation inhibitors (DMIs) and newer fungicides such as strobilurins.

The sensitivity reduction of *V. inaequlis* isolates, collected from west and south Turkey, to benzimidazoles, DMIs and dodine were reported in 90s (Benlioğlu and Kılıç, 1994). In another study, the effectiveness of different doses of 14 fungicides licenced against apple scab were tested *in vitro* and *in vivo* in 2008-2009 years. Among the tested fungicides *in vitro*, the mycelium development of apple scab was full inhibited at 10 ppm concentration for mancozeb, 50 ppm for dodine and captan, and 100 ppm for pyraclostrobin+dithianon and pyrimethanil. Other active ingredients effected the development at the different levels. In field trials, the differences between replications, months and years were found unimportant

statistically. The most effective fungicides were determined as dodine (84.63%), pyraclostrobin+dithianon (66.07%), captan (64.62%) and mancozeb (60.85%) in the mean of 2008 and 2009 years (Kaymak *et.al*, 2011).

Strobilurins, due to their efficacy against several important plant diseases and wide working range in different weather conditions, constitute the most attractive group of fungicides among fungicides used in Turkey. Strobilurins have been used here since 1998 (Table 2). After few of intensive treatment (up to 4–5 applications per season), the appearance of *V. inaequalis* forms with reduced sensitivity to strobilurins were seen in apple orchards. Strobilurins continue to be used in most Turkish apple orchards, often with mixed with products characterized by other mechanisms of action. However, the molecular mechanism responsible for QoI resistance in Turkish orchards has not been studied yet.

Table 2. Authorized strobilurins on apple scab in Turkey

Strobilurins	Dose	Formulation	Authorization Date
kresoxim-methyl	15g/100 L	WG	13.03.1998
trifloxystrobin	15g/100L	WG	15.02.2000
pyraclostrobin (12.8%) +boscalid (25.2%)	30g/100L	WG	17.03.2006
metominostrobin (200g/L)	60ml/100L	SC	23.05.2006
pyraclostrobin (4%) + dithianon (12%)	100g/100L	WG	14.06.2007

2.6 Strobilurins

Since strobilurin's first launched in 1996, sales of strobilurin fungicides have enjoyed an average annual growth of 15.7% in five years (Fig 8). Registrations have been obtained on a wide range of crops throughout the world and reached to $1.636 billion in 2007 as a result of widespread use (Morton and Staub, 2008). The success story of strobilurins as

fungicides is due to their characteristics, which are broad-spectrum activity, control of fungal isolates resistant to other fungicides modes of action, low use-rates, and excellent yield and quality benefits (Sauter *et al*. 1999). They have been used as foliar fungicides, for seed treatment, as well as in-furrow treatments for soil-borne diseases.

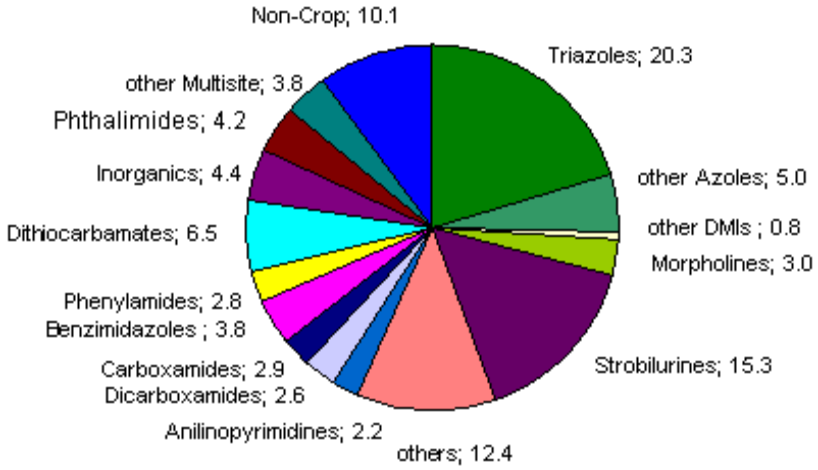


Fig.8. Percentage of fungicides in the world market of fungicides for 2005. (Phillips McDougall, 2006)

The first development azoxystrobin and kresoxim- methyl were announced, respectively, in 1992 and they were sold the first time in 1996 for the control of diseases in cereals (Margot *et al*., 1998; Barlett *et al*., 2002) and then few years later metominostrobin and trifloxystrobin were sold in 1999. Picoxystrobin and pyraclostrobin were announced and sold in 2002. During the 1990s also famoxadone and fenamidone were discovered. Famoxadone and fenamidone are two additional fungicides that are chemically distinct but share the same crossresistance group with the strobilurins.

Other QoI fungicides, coumoxystrobin, dimoxystrobin, enoxastrobin, fenaminostrobin, fluoxastrobin, flufenoxystrobin, orysastrobin, pyraoxystrobin, pyrametastrobin, pyribencarb, triclopyricarb are also in the same cross-resistance group (FRAC, 2011).

2.6.1 Mode of action of QoI Fungicides

Strobilurin fungicides were created by a group of natural fungicidal derivatives of β-methoxyacrylate acid (Becker *et al.,* 1981). They are developed from the natural compound strobilurin A, oudemansin A and myxothiazol A. These natural products are a secondary metabolite of the Basidiomycete wood-rotting fungi, such as *Qudemansiella mucida* (Schrad ex Fr) Hoehn, *Strobilurus tenacellus* (Pers ex Fr) Singer and, in the case of myxothiazol A, the gliding bacterium *Myxococcus fulvus* (Barlett, 2002).

Strobilurins have a single-site mode of action that inhibit fungal respiration by binding to cytochrome b of complex III at the Q_o site in the mitochondrial respiration chain (Fig 9). Therefore they are known as Qo inhibitors (QoIs) (Sauter *et al*., 1999). This results in the disruption of adenosine triphosphate (ATP) production resulting in energy deficiency in the fungus. Since they disrupt ATP/energy production in phytopathogenic fungi, they greatly affect the fungal stages that require large amounts of energy. These stages are spore germination and zoospore motility (Bartlett *et al*., 2002). They have also been observed to inhibit the formation of mature cleistothecia in *Erysiphe necator* and formation of mature oospores of *Plasmopora viticola* and to reduce their viability (Bartlett *et al.* 2002).

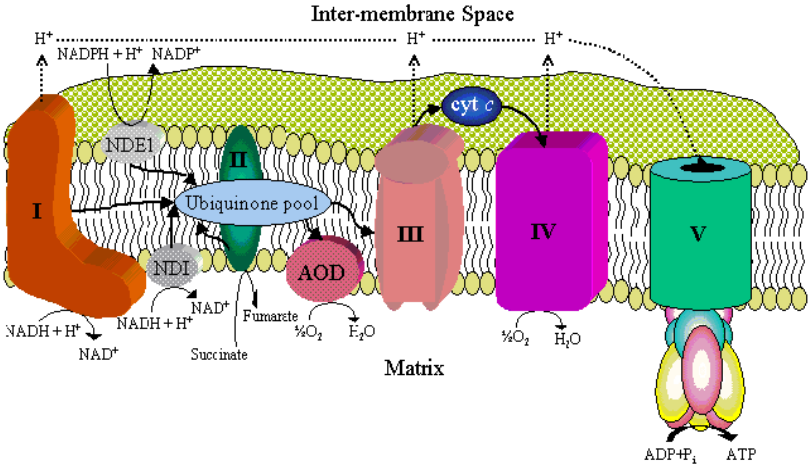


Fig. 9. The mitochondrial respiratory chain. (from http://pages.slu.edu/faculty/kennellj/)

Field application of the natural compounds of strobilurins was not possible because of their relative volatility and photochemical instability. Also they beak down rapidly in light and are therefore not reliable for disease control.

Therefore, efforts were directed at chemical optimization and a knowledge of their structures and physical properties provided to develop the synthetic analogues, QoI fungicides (Sauter *et al*., 1999). The various QoI fungicides have very different physicochemical properties which confer different behaviors in the plant.

Table 3. Properties of strobilurins

	Azoxystrobin	**Trifloxystrobin**	**Kresoxim -methyl**	**Metominostrobin**	**Pyraclostrobin**	**Picoxystrobin**
Movement into leaf	low	Very low	low	high	Very low	Medium
Vapour active	no	yes	yes	no	no	Yes
Metabolic stability in leaf	yes	low	low	n.d.*	yes	Yes
Translaminar movement	yes	low	low	yes	low	Yes
Xylem systemic	yes	no	no	yes	no	Yes
Systemic movement to new groth in creals	yes	no	no	yes	no	Yes
Phloem mobile	no	no	no	no	no	No

Source Syngenta; *n.d. = no data (Bartlett *et al*. 2002).

Azoxystrobin and picoxystrobin retain the methyl β-methoxyacrylate group of the natural fungicides, while the others contain modified toxophores. The fungicide azoxystrobin and picoxystrobin move translaminarly as well as systemically. The fungicides kresoxim methyl, trifloxystrobin and pyraclostrobin move translaminarly but not systemically. Azoxystrobin, picoxystrobin and metominostrobin are xylem-systemic (Table 3). In addition, picoxystrobin is the most rapidly absorbed into plant tissue. 30-45% of material

applied to the foliar surface is absorbed into the cells of the leaf of wheat and barley within 24h of application. Also kresoxim-methyl, picoxystrobin and trifloxystrobin deliver disease control in the vapour phase, but azoxystrobin and pyraclostrobin can not be redistributed in the same way (Bartlett *et al*., 2002).

2.6.2 Spectrum of disease control

QoIs control plant diseases caused by pathogens from all groups of fungi: Oomycetes, Ascomycetes and Basidiomycetes. They are used on a wide variety of crops, including cereals, field crops, fruits, tree nuts, vegetables, turfgrasses, and ornamentals against pathogens such as *Venturia inaequalis*, *Podosphaera leucotricha*, *Mycosphaerella fijiensis*, *Phytophthora infestans*, *Alternaria solani*, *Pythium* spp., *Rhizoctonia solani*, *Microdochium nivale*. QoIs are recommended for the foliar diseases of cereals and rice, such as *Mycosphaerella graminicola*, *Pyrenophora teres*, *Erysiphe graminis*, *Puccinia recondita*, *Puccinia hordei*, *Rhynchosporium secalis* and *Pyricularia grisea*.

Azoxystrobin is recommended for use as a foliar, submerged, seed or soil treatment, Azoxystrobin controls blast (*Pyriculuria oryzae*) and sheath blight (*Corticium sasaki*i) on rice. It has a very good activity against grape downy mildew (*P. viticola*) and powdery mildew (*E. necator*), and *Alternaria mali*, and potato late blight (*P. infestans*). Also it is commercialized for the control of brown patch (*Rhizoctonia solani*), Pythium blight (*Pythium* spp.) and pink snow mould (*Microdochium nivale*) of turfgrass (Gullino *et al*., 2000).

Trifloxystrobin has a very broad spectrum of activity, such as against powdery mildews on cucurbits and grapevines and leaf spot diseases like *V. inaequalis*, *V.pirina*. It also significantly controls of rusts, downy mildews and alternaria diseases.

Kresoxim-methyl has also some efficacy on a broad range of pathogens and is registered mostly on cereals, especially for powdery mildew (*E. graminis*), net blotch (*Drechslera teres*), and *Septoria nodorum*. It also shows excellent eradicant properties against *E. graminis* and *E. necator.* Generally, kresoxim-methyl is more effective against spore germination than mycelial growth because of the spore's dependency on mitochondrial respiration during germination especially for *V.inaequalis, P. oryzae* and *R. secalis* (Sauter *et al.*, 1995).

Metominostrobin has excellent activity against rice blast and sheath blight, powdery mildew of vegetables and cereals, scab and rust of apple and pear, grey mould and Sclerotinia rot of vegetables.

Picoxystrobin is recommended for a range of cereal diseases.

Strobilurins are best used in pre infection period or in the early stages of disease development because of the their potent effects on spore germination and zoospore mobility (Godwin *et al.*, 1994; Godwin *et al*., 1997; Stierl *et al*., 2000). However, curative activity has also been observed. This has been found with azoxystrobin, trifloxystrobin, kresoxim-methyl, pyraclostrobin and picoxystrobin (Barlett *et al*., 2002). In addition, some studies have been demonstrated that azoxystrobin inhibits the formation of mature cleistothecia of grapevine powdery mildew (*E. necator*) and the production of visible ascosopores (Godwin *et al*., 1999; Bartlett *et al*., 2002).

2.6.3 Mechanisms of resistance to QoI Fungicides

Soon after introducing QoI inhibitors to the market in 1996, the development of resistant forms in field populations of several important plant pathogens has been reported in different countries (Barlett *et al.*, 2002; Gisi *et al*., 2000).

In 1998, wheat powdery mildew (*E. graminis* f.sp. *tritici*) resistant isolates to QoI fungicides were first detected in Northern Germany (Felsenstein, 1999). QoI resistance has been also detected in other pathogens, including *Sphaerotheca fuliginea* in Asia and parts of southern Europe (Heaney, 2000) and *Pseudoperonospora cubensis* on cucumber in Spain and Japan (Ishii *et al*., 1999), *P. viticola* on grape in Italy and France, and *Mycosphaerella fijiensis* on banana in Costa Rica (Heaney, 2000; Gisi *et al*. 2002).

QoI resistance in *V. inaequalis* was first reported in 1997 in European field and experimental trials in Switzerland and Northern Germany (Fontaine *et al*, 2008; Farber *et al*., 2002; Steinfeld *et al.*, 2002). Also several cases of reduced apple scab control by strobilurins were reported in Chile (Sallato *et al*. 2006), in Italy (Fiaccadori *et.al*, 2005, 2011), in Poland (Broniarek-Niemiec and Bielenin, 2008) and in other European countries (Fig 10) (Frac, 2011).

A commonly observed mechanism of resistance in many phytopathological fungi is single-nucleotide mutation in the cytochrome b gene, leading to amino acid exchange and preventing the inhibitor from binding to the respective gene product (Lesemann *et al*., 2006). There are three most common among amino acid substitutions in several phytopathogenic fungi and oomycetes resistant to QoIs: from glycine to alanine at position 143 (G143A), from phenylalanine to leucine at position 129 (F129L) (Gisi *et al*., 2002) and from glycine to arginine at position 137 (G137R) (Sierotzki *et al.*, 2006).

The QoI-targeted cytochrome b protein is encoded by mitochondrial DNA (mtDNA). This DNA generally is thought to mutate at a higher frequency than that of nuclear DNA (Ishii *et al*., 2007). This genetic trait must have greatly influenced the rapid development of resistance to QoI fungicides.

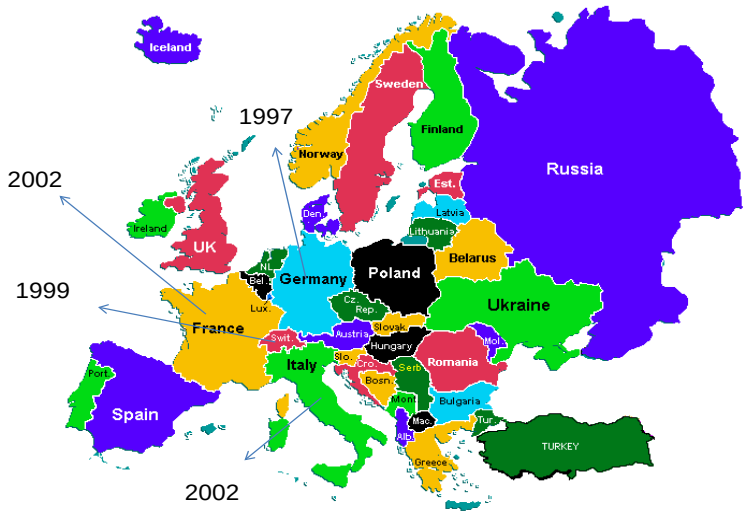


Fig.10. The first appearance of QoI resistance in *V. inaequalis* in European countries

In 2000, Sierotzki *et al*. (2000a) detected for the first time the presence of G143A substitution in cytochrome b gene in QoI-resistant field isolates of plant pathogens such as *Blumeria (Erysiphe) graminis* f.sp. *tritici* on wheat and *Mycosphaerella fijiensis* on banana (Sierotzki *et al*., 2000b). This target site mutation has been identified in more than twenty species, including phytopathogenic Ascomycetes and oomycetes such as *V. inaequalis* (Zheng *et al*., 2000), *Botrytis cinerea* on strawberries, several powdery mildews and *Alternaria* species, major *Mycosphaerella* pathogens (Fungicide Resistance Action Committee, FRAC, QoI Working Group)[www.frac.info].

In *V. inaequalis*, the G143A mutation was detected for the first time in Northern Germany in 1999, and in the following years also in Poland (Barlett, 2002), and Northern Italy in 2002 (Fiaccadori *et al*., 2005, 2011).

In addition to the G143A mutation, a QoI-resistant mutant of *Magnaporthe grisea* created in a laboratory had a G143S (glycine to serine) mutation at the *cyt b* gene. Both

G143A and G143S mutations remained stable during four consecutive disease cylces in the absence of axozystrobin (Ma and Michailides, 2005).

The F129L mutation confers a lower resistance level than G143A (Bartlett *et al*., 2002; Sirven and Beffa 2003), often occurs at lower frequencies in a sample when both mutations are present (Sierotski *et al*., 2005). This second mutation was detected in *Pyricularia grisea* and *Pythium aphanidermatum* which are turfgrass pathogens (Bartlett *et al.*, 2002; Grasso *et al*., 2006), *Rhizoctonia solani AG1.1A* on rice (FRAC, 2011), *Alternaria solani* (Sierotski *et al*., 2005), *Pyrenophora teres* and *P. viticola.*

Recently, the G137R mutation has been identified only in *Pyrenophora tritici-repentis* (Tan Spot) on wheat. A complete and updated list of plant pathogens that developed resistance to strobilurins can be found in the FRAC website (Fungicide Resistance Action Committee, FRAC, QoI Working Group)[www.frac.info], 2011).

The sequences of the two regions of cytochrome b spanning the Qo site from Strobilurin-producing fungi, such as *Strobilurus tenacellus, Mycena galopoda*, was compared to understand the molecular basis of natural resistance to strobilurins. Five amino acid changes in cytochrome b were found. These substitutions are: T127I (threonine changes to isoleucine), A153S (alanine changes to serine), S255Q (serine changes to glutamine), N262D (asparagine changes to aspartic acid), and G143A (glycine changes to alanine). That's why it is not surprising that the main point mutation (G143A) developed by phytopathogenic fungi which confers field resistance to QoI (Fernandez-Ortuno, 2008).

The second resistance mechanism involves the activation of the alternative respiration pathway in the electron transport chain of the mitochondria by means of expression of an alternative oxidase (AOX) gene. With this rescue mechanism, mitochondrial electron transfer is diverted by circumventing the inhibitory site of QoI, the cytochrome bc1complex. This alternative has been found to be active in mycelial growth and

conidia germination *in vitro* in the presence of strobilurins, but this activity has been considered of little importance under field conditions. Also AOX is less efficient in oxidation than the Qo site of the mitochondrial complex III and this pathway provides only 40% of the normal efficiency for energy conservation (Fernandez-Ortuno, 2008). That's why when the Qo site has been blocked by a strobilurin, AOX is probably insufficient for fungal survival during periods of high metabolic activity, such as spore germination and plant infection. The second reason of ineffectiveness of alternative respiration is plant antioxidants. During infections, they are released and interfere with the induction of alterative respiration (Fernandez-Ortuno, 2008). Salicylhydroxamic acid (SHAM) has used to inhibit the activity of AOX during strobilurin resistance testing studies (Olaya and Köller 1999a, 1999b; Steinfeld *et al*., 2001).

Jabs *et al*. (2001) reported that the partial loss of control of kresoxim- methyl had been caused by an external esterase that was able to metabolise the fungicide. Studies showed that the esterase was specifically effective to cleave the ester bond in the toxophore of kresoxim- methyl but was much less effective in the case of other strobilurins. In plantations where kresoxim-methyl showed some efficacy losses due to metabolisation, trifloxystrobin always showed a perfect control. So, this resistance mechanism seems to be specific for kresoxim-methyl (Kuck and Mehl, 2003).

2.7 New approaches for sensibility monitoring studies towards QoI Fungicides

Monitoring the presence of QoI-resistant isolates and the level of mutated allele in field pathogen populations is important for studying resistant evolution and the practical evaluation of strobilurin efficiency in the corresponding orchards. However, detection of *V. inaequalis* QoI-resistant isolates relies on *in vitro* test systems based on spore germination on fungicide-amended media. Biological assays are widely used for resistance screening among fungal populations. Unfortunately, they can be labor-intensive and time-consuming if large

numbers of isolates are to be tested and also the viability of *V. inaequalis* conidia recovered from commercial orchards treated with pesticides is usually low.

Once, the resistance mechanism is known, it is often faster to use molecular methods. The development of molecular methods has provided to detect rapidly fungicide resistant genotype, therefore classical methodology is often supplemented with new molecular tools, which can be used in parallel or even instead of bioassays in various application fields (Michalecka *et al*., 2011).

PCR–RFLP, DHPLC hybridization (Baumler *et al*. 2003), AS-PCR (Allele-Specific PCR) and CAPS (Cleaved Amplified Polymorphic Sequence) assay (Fontaine *et al*. 2008; Fiaccadori *et al*., 2005, 2011) have been used successfully to detect *V. inaequalis* resistance genotypes.

The recent development of quantitative Real-Time PCR (q-PCR) allows to detect the frequency of a resistant allele in a DNA sample. qPCR allows for detection of the mutation even when it is present at very low frequencies. qPCR is a very powerful, cultivation-independent, rapid, sensitive and useful tool, it has been used to detect several organism in a wide range of research fields.This method can help growers to control or manage diseases effectively. qPCR method has also been used to quantify the G143A substitution that confers QoI resistance in *Alternaria* populations, *B. graminis*, *P. viticola* and *E. necator* (Ma and Michailides, 2005; Dufour *et al*., 2010).

Recently, quantification of QoI resistant allele in *V. inaequalis* populations has been developed (Nanni *et a.l*, 2011; Michalecka *et al*., 2011).

2.8 Aim of the study

The monitoring of QoI resistant populations through biological tests and the molecular analysis is important to demonstrate the efficacy of strobilurins application and the evolution of resistance.

The main goal of this study was to correlate the results of *in vitro* tests with those of q-PCR on a wide range of *V. inaequalis* populations and monoconidial isolates. They were collected in Italian and Turkish distinct locations from orchards with different scab management.

The other goals of the research conducted for this thesis were:

- to determine the sensitivity to trifloxystrobin of populations and monoconidial isolates using conidial germination assays and mycelium growth assay on fungicide amended agar plates;

- to determine the presence of G143A substitution by Cleaved Amplified Polymorphic Sequence (CAPS);

- to develop a method that is able to quantify the percenteage of mutated allele determined by a substitution G143A and to apply this method on *V. inaequalis* field populations and their monoconidial isolates.

3. Materials and methods

3.1. Collection and maintenance of fungal population and monoconidial isolates

Populations

55 bulk *Venturia inaequalis* populations in northern Italy orchards during 2002-2011 and 9 bulk populations in western and southern Turkey orchards in 2011 were sampled. *V. inaequalis* populations were sampled in apple trees with different origin and scab management: wild-types, untreated and ones treated with several groups of fungicides, comprehending often strobilurins both with good or poor exit control (Table 4). Samples consisted in 40-50 scabbed leaves randomly collected in each orchard in May, June and July. Upon arrival in the laboratory, samples were immediately examined using a dissecting microscope to determine the germinability of sporulating *V.inaequalis* conidia.

In order to obtain populations with high germinative energy, a drop of sterile water was put on each of 30- 40 scab lesions and conidia were harvested by rinsing with sterile water. The conidial concentration was adjusted to 1-3 x 10^5 spores mL and inoculated on potted apple seedlings in greenhouse. Inoculated seedlings were incubated for a further 15-20 days at 21 ^oC in greenhouse. Scabbed seedling leaves were then conserved in silica gel at 4 ^oC for sensitivity tests. Also 15-20 scab leaf discs were prepared by a sterile cork borer (9mm diameter) for each population to use in molecular analysis and stored at -20 ^oC until DNA extraction.

Table 4. Sampled *Venturia inaequalis* populations with different apple scab control in Italy and in Turkey

Origin of population	Sample year	Population ID	Strobilurin Management[1]		Origin of population	Sample year	Population ID	Strobilurin Management[1]
Italy	2002	1202	WT		Italy	2009	506	PC
	2003	12III	WT			2009	507	PC
	2003	62	WT			2009	508	PC
	2003	96	GC			2009	512	PC
	2003	87	PC			2009	523	PC
	2003	88	PC			2009	533	PC
	2004	102	PC			2009	535	PC
	2004	115	UNT			2009	543	PC
	2005	122	WT			2009	546	PC
	2005	130	BIO			2009	549	PC
	2005	136	WT			2009	550	PC
	2005	138	PC			2009	551	PC
	2005	144	PC			2010	600	NOS
	2005	156	GC			2010	602	PC
	2005	158	WT			2010	605	PC
	2005	161	PC			2010	611	PC
	2006	201	NOS			2010	612	ND
	2006	202	GC			2010	616	PC
	2006	225	PC			2010	BA3	NOS
	2006	226	WT		Turkey	2011	700	WT
	2006	228 I	WT			2011	701	PC
	2006	229	PC			2011	702	PC
	2007	302	PC			2011	703	PC
	2007	307	PC		Italy	2011	706	PC
	2007	319	PC			2011	707	PC
	2008	408	PC			2011	708	PC
	2008	411	PC		Turkey	2011	709	UNT
	2008	412	PC			2011	710	UNT
	2008	426	WT			2011	711	UNT
	2008	427	GC			2011	712	UNT
	2009	503	PC			2011	713	UNT
	2009	504	PC		Italy	2011	714	PC

[1] WT: wild-type, UNT: untreated, GC: good control of strobilurins, PC: poor control of strobilurins, NOS: no strobilurin used, BIO: biological orchard, ND: information not available

Monoconidial isolates

On the basis of *in vitro* results of populations and their origin, some populations (62, 1202, 156, 202, 102, 503) were chosen to obtain their monoconidial isolates. Conidia were harvested from about 20 lesions per sample by rinsing with sterile water; the suspension was diluted to 20–30 conidia/microscope field (100×). Suspension aliquots of 0.2mL were

streaked on petri dishes of water agar (2% agar grade A; Becton, Dickinson and Company, Sparks,MD) amended with 400 mgL^{-1} of streptomycin sulfate. After 24 h incubation at 20 °C, single germinated spores were selected under stereomicroscope, drawn and placed on PDA (3,9%; Becton, Dickinson and Company) amended with 300 mg L^{-1} of streptomycin sulfate, chlortetracycline, chloramphenicol. After 60 days, the colonies were ready for conidial production, *in vitro* sensitivity assays and molecular analysis (Fig 11).

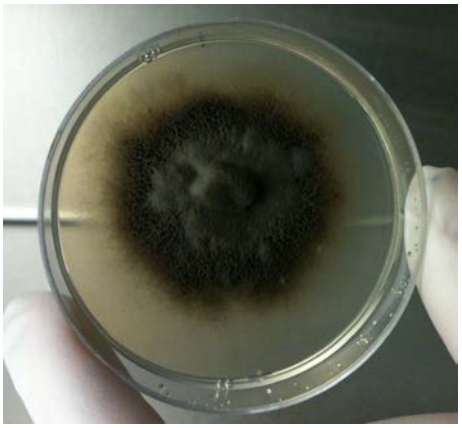


Fig. 11. Single germinated spore of *V. inaequalis* after 60 days

3.2 Biological assays

In vitro assays were carried out in petri dishes and concerned the conidial germination for the populations and monoconidial isolates. Also, the mycelial growth test was evaluated for the monoconidial isolates.

3.2.1 *In vitro* sensitivity assays of populations

Trifloxystrobin (Sigma-Aldich, St Louis,MO) was used as active material. The stage of conidia germination was chosen because trifloxystrobin was described as primarily active in a protective mode of apple scab control (Olaya and Koller, 1999b).

Trifloxystrobin was solubilized in acetone (concentration in final solution <0.01 mL mL^{-1}) and added to water-agar (2% agar grade A; Becton, Dickinson and Company) to

achieve the following concentrations: 0.0001, 0.001, 0.01, 2 mg L^{-1}. The antibiotic streptomycin sulfate was added to water agar at a final concentration of 200 mg L^{-1}.

In each *in vitro* test, pieces of scabbed leaves (0.012-0.020 g) were randomly collected from each sample and introduced in micro tubes with 1 mL of sterile water. After shaking, the conidial concentration was adjusted to 1-3 x 10^5 spores mL^{-1}. Two drops of 20 µL of spore suspension for every fungicide concentration were placed on agar plates and incubated for 24 hours at 20 °C. For every concentration two or three replicates were prepared. 150 conidia for replicate were visually assessed at the microscope, counting those that had germinated.

Data were trasformed in EC_{50} value by probit analysis. Generally, 2-3 *in vitro* tests were performed for each population, and mean EC_{50} values (the concentration that inhibits 50% of spore germination compared with an unamended control) of every population were calculated. Furthermore, according to test results, percentage of germination at the highest concentration (2 mg L^{-1}) and relative germination (RG) (percentage of germination at maximum concentration/percentage of germination in nontreated x 100) were also calculated to correlate with percentages of mutated alleles by qPCR. The correlation was assessed with analysis of variance (P value, coefficient of correlation, confidence level and r^2) by Statgraphics Plus v.1 program.

The same data analyses were used for all *in vitro* tests.

At the beginning of strobilurin resistance tests, the alternative respiration inhibitor salicylhydroxamic acid (SHAM) at 100 mg L^{-1} was added to trifloxystrobin at different concentrations to inhibit the activity of alternative oxidase (AOX). However, the addition of SHAM didn't increase the activity of trofloxystrobin for populations and moonoconidial isolates (Table 6 and 9). Therefore, based on these results, fungicide sensitivity assays for populations and monoconidial isolates with trifloxystrobin were conducted without adding SHAM.

3.2.2 *In vitro* sensitivity assays of monoconidial isolates

In vitro sensitivity assays of monoconidial isolates were carried out by mycelium growth and conidial germination test. From 8 to 13 monoconidial isolates were tested for some of the populations with a higher sensitivity (n.1202, 62), less sensitivity (n.202, 156) and reduced sensitivity (n.102, 503) in conidial germination tests.

Mycelium growth test

For every isolate, a 4mm mycelium plug was placed on PDA (3.9%; Becton, Dickinson and Company) amended with different concentrations of trifloxystrobin according to the methodology previously described (Fig 12). The mycelial growth was measured 20 days later as colony diameters (minus the diameter of the plug). The EC_{50} values were calculated using probit analysis.

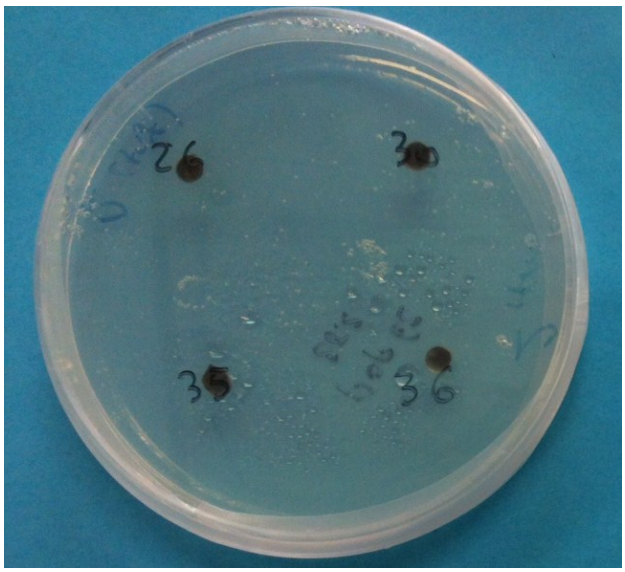


Fig. 12. 4mm mycelium plug of monoconidal isolates on PDA amended with trifloxystrobin

Conidial germination tests of monoconidial isolates

Conidial production method of Venturia inaequalis

Conidia were produced from mycelia of monoconidal isolates. Cellophane-covered surfaces of PDA were used for high yields of conidia of *V. inaequalis.* (Parker *et al.* 1995).

Cellophane Preparation:

Cellophane membranes were cut in 8.0cm disks and soaked in 3L of water (HPLC Grade) for 12 h. Soaked disks were layered between 9.0cm diameter filter paper and placed in a glass petri dish. The dish was filled with water (HPLC grade) and autoclaved for 15 min at 120°C. Then the sterile cellophanes were layered onto PDA (3,9%; Becton, Dickinson and Company) avoiding the formation of air spaces.

Mycelium of monoconidial isolates was derived from fungal cultures grown on PDA for 8-9 week (20-40 mm colony diameter). Mycelium of one colony was removed from the agar surface and placed in a sterile tube with 6 ml sterile water. The tube was centrifuged with vortex for 10x10x10x10 s with pauses (5 s). Suspension of blended mycelium was transferred to the sterile container by sterile filter. Generally, the conidial concentration was observed 1-10 x 10^5 spores mL^{-1}. A suspension of conidia was transferred to the cellophane-covered agar surface. The surface is seeded with a suspension of conidia at various densities (0.8 ml-1 ml for one Petri dish). The dish was tripped until the suspension was evenly distributed across the surface and was sealed with parafilm and incubated in the light were placed in a single layer at a distance of 25cm from a continuous light source.

After incubation for 2 to 4 week (Fig 13), the cellophane was removed from the agar surface and placed in a 150 ml beaker containing 50 ml water. The beaker was stirred rapidly for approximately 5 minutes to remove the conidia from the cellophane. The conidial suspension was poured through filter in another beaker in order to remove residual mycelium. The number of conidia was determined with Thoma counting chamber and the conidial concentration was adjusted to 1-3 x 10^5 spores mL^{-1} for in vitro test. Before their

insemination on amended petri dishes, germinated conidia were observed but the number of germinated conidia did not exceed 10%. Spore suspensions for every fungicide concentration were placed on agar plates. The sensitivity of germinating conidia was tested and results were calculated as described in *in vitro* test of populations.

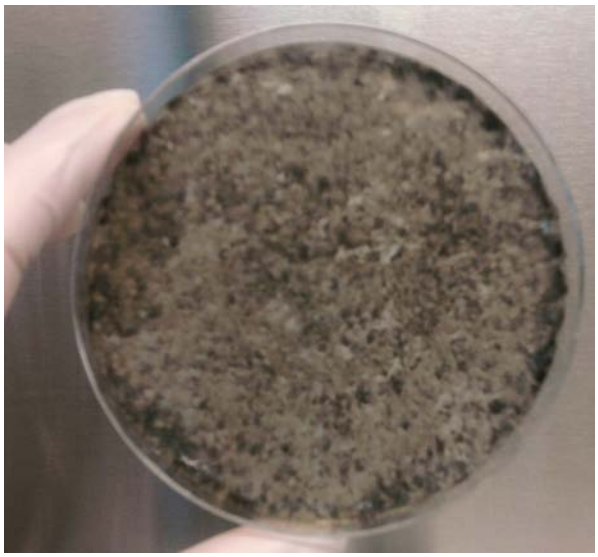


Fig. 13. The cellophane covered with *V. inaequalis* conidia and mycelium

Same spore suspensions were used for inoculation on apple seedlings to evaluate the conidial germination test after obtained conidia on leaves.

Same spore suspensions were also stored at -80 °C until molecular analysis.

In vitro tests with inoculated leaves by monoconidial isolates

Same spore suspensions of some monoconidial isolates which were used for conidial germination tests inoculated on apple seedlings to test the sensitivity of germinating conidia in order to conserve the sporulation in dried ambient and avoid the anticipated germination. Inoculations were realized by obtaining conidia suspensions of 1-3x10^5 spores mL^{-1}, spraying 1.2 ml per young apple seedlings with no more than 5-6 leaves. Inoculated seedlings were incubated for a further 15-20 days at 21°C in greenhouse. In each *in vitro* test,

pieces of scabbed leaves (0.012-0.020 g) were randomly collected from each sample and introduced in micro tubes with 1 mL of sterile water. After shaking, the conidial concentration was adjusted to 1-3 x 10^5 spores mL^{-1}. The sensitivity of germinating conidia was tested and results were calculated as described in *in vitro* test of populations.

3.3 Molecular analysis of populations and monoconidial isolates

3.3.1 DNA extraction of population and monoconidial isolates

15-20 scab leaf discs were prepared by a sterile cork borer (9mm diameter) for each population to use in molecular analysis and stored at -20^{o}C until DNA extraction. The genomic fungal DNA was extracted directly from the scabbed leaves which were ground in liquid nitrogen by a CTAB - based method (Murray and Thompson, 1980) and then purified through a Sepharose (Sigma-Aldrich, St Louis, MO). Amount, purity and integrity of DNA samples were assessed on the basis of an absorbance ratio of 1.80 -1.90 at 260/280 nm and of 1.90-2.30 at 260/230 nm using NanoDrop ND1000.

In order to obtain the genomic fungal DNA of monoconidial isolates, spore suspensions of monoconidal isolates that tested in *in vitro* tests were used as extraction material utilizing Chelex 100 chelating resin (Sigma). The Chelex extraction method has been developed for extracting DNA from forensic-type samples for use with the PCR. (Walsh *et al*., 1991). Some monoconidal isolates were extracted also by a CTAB - based method to compare the effectiveness of Chelex method. DNA of monoconidial isolates obtained using the two different extraction methods were identical for all samples tested. Finally, chelex method was chosen because it is a fast, cheap, involve no organic solvents and effective method for less DNA material.

It is a new exctraction method for *V. inaequalis* DNA material which is developed by our research group.

Chelex 100 Protocol

Preparation of 10% Chelex 100 Aliquots stock solution :

1) Obtain a new 50 mL polyethylene conical tube

2) Fill the conical tube to the 40mL mark with dH_2O

3) Weight out 4 grams of Chelex 100. Pour into falcon tube

- 1-3 x 10^5 spores mL^{-1} were added to aliquot 300 µl of 10% Chelex (Sigma) solution in a 1.5-ml microcentrifuge tube
- Samples were vortexed for 2 minutes and spinned briefly at 12.000rpm in a micro centrifuge for 10-15 seconds
- Samples were incubated at 90°C for half an hours, vortexed for 2 minutes and spinned at 12.000rpm again.
- Samples were incubated again at 90°C for 15 minutes.
- The supernatant DNA was transferred to another tube and purified through a Sepharose (Sigma-Aldrich, St Louis, MO) and stored at -20°C until molecular analysis.

Amount, purity and integrity of DNA samples were assessed on the basis of an absorbance ratio of 1.80 -1.90 at 260/280 and of 1.90-2.30 at 260/230 nm using NanoDrop ND1000.

3.4 Qualitative Analysis

3.4.1 Cleaved amplified polymorphic sequence (CAPS) Analysis

The molecular analyses were performed after DNA extraction of all populations and monoconidal isolates. A 413 bp fragment of cytochrome *b* of *V. inaequalis*, including the region codifying the G143A site, was then amplified using the primers ANK 10 reverse (5'-CTG TTG TTA GGC TCT TCA ATG -3') and ANK 283 forward (5'-CTG TAG TTG AAA

GGC TAT TAG -3‘). These primers were specific for *V. inaequalis*, as no amplification was observed with DNA of other fungi such as *Venturia pyrina* Aderh. and *Alternaria* species [*A. Mali* Roberts, *A. alternata* (Fr.) Keissler]. PCR amplifications were performed in a ICycler (Bio-Rad, Hercules,CA) in 50 µL of mix reaction containing 5 µL of reaction buffer 10×(Takara, BIO INC,Japan), 0.2 mM of dNTPs, 0.25 µM of each primer, 1 µl of total DNA (20ng/ µl) and 1.25 units of rTaq DNA polymerase (Takara, BIO INC.Japan). The reaction started at 94°C for 2 min and continued for 40 cycles at 94°C for 45 s, 57°C for 45 s and 72°C for 45 s. The final extension was at 72°C for 7 min. PCR products were then digested with *Tse* I restriction enzyme (New England BioLabs, Ipswich, MA) which is able to recognise its target site only when the mutation causing G143A substitution is present. After electrophoresis, the agarose gel (1.5%) was stained with ethidium bromide, and the sizes of DNA fragments were determined by comparison with the 100 bp DNA ladder (New England BioLabs).

3.5 Quantitative Analysis

3.5.1 Cloning of sensitive and resistant strains of *Venturia inaequalis*

With the aim to obtain both, the resistant and sensitive strains a CAPS PCR with monoconidial isolates of *V. inaequalis* was performed. Sequencing reactions were done by Sanger methods using gene-specific primers (BMR Service, Italy). A BLASTN search was carried out in the NCBI database to identify correct strains. After PCR, excess primers were removed using GenElute PCR Clean-up kit (Sigma-Aldrich) and the specie-specific PCR product ligated directly into the pGEM-T easy vector (Promega Corporation, Madison, WI, USA). Plasmids were transformed into *Eschirichia Coli* JM109 cells (Promega, Madison WI,USA) according to a standard protocol, and plasmid DNA was extracted using Quick

Plasmid Miniprep Kit,(Invitrogen, Carlsbad, CA) according to a manufacturer protocol. For further confirmation direct sequencing of sensitive and resistant clones was done.

3.5.2 Quantitative qPCR analysis

Primers set-up - The G143A mutation in *Venturia inaequalis* population and monoconidial isolates were performed using a qPCR method. A specific primers set were set-up. The forward primers FwS5418 and R5548 were used respectively for sensitive and resistant allele, while reverse common primer, R5548, was used (Table 5). With the aim to verify the qPCR efficiency (E) and specificity of each primer pair, different percentage of referent sensitive and resistant plasmidial DNA were analyzed: 100 % Senstive (S), 1 % Sensitive (S) + 99 resistant (R), 5 % Sensitive + 95 % Resistant (R), 10 % Sensitive (S) + 90 % Resistant (R),50 % Sensitive (S) + 50 % Resistant (R), 99 % Sensitive (S) + 1 % Resistant (R),100 % Resistant (R).

% The ratio of the two alleles presence in samples was calculated using ΔC_q method (Bustin *et al.*, 2009) according the equation:

10^ (Cq sensitive – Cq resistent)/slope), = R (R/1+R) x 100 = R allele;

Where *Cq* is the quantification cycle and reflects the cycle number at which the fluorescence generated within the reaction of each sample crosses the threshold and the slope was calculated according to the standard curves, which were specific for each allele. The data was expressed as percentage of resistant of sensitive allele.

Standard curves development - *Standard curves* were obtained using sensitive and resistant, mixed strains (50% sensitive and 50% resistant) 10-fold serial dilutions ranging from 20 ng $x10^{-3}$ to 20 ng $x10^{-6}$ of plasmid DNA. The standard curve efficiencies were; sensitive E= 101.9%, resistant E= 100.1%.

The high efficiency for each gene allowed the assumption that the genes are amplified with the same efficiency, and an average slope of – 3.298 was used in the equation.

Quantitative qPCR conditions

The qPCR analysis of 64 *V. inaequalis* populations and 60 monoconidial isolates was performed using ICycler (Bio-Rad, Hercules,CA) The reaction mixture was prepared in a final volume of 20 µl, including 12 µl of 2 × SYBR Green I (Bio-Rad), 1 µl of forward and reverse primers (12.5 µM each), and 8 µl of diluted DNA (1/5).

Amplifications were performed in 96-well Hard-shell PCR plates (Bio-Rad, Hercules, CA,USA) The following thermal cycling conditions were used: one cycle at 95°C for 3 min followed by 40 cycles at 95°C for 18 s, 55°C 12 s, and 72°C for 18 s.

To verify the specificity of the product obtained, a melting curve was performed at the end of the PCR reaction with an increase of the temperature specificity of 0.05°C/s, from 55 to 95°C. The assays are carried out in duplicate, and each experiment was repeated two or three times. Data are calculated using the supplied Real-Time Detection System software version 3.0 for Windows (BioRad) according to the manufacturer's instructions.

Table 5. Primers sets used for qPCR

Primer Name	**Orientation 5' -3'**	**Primer Sequence**
5418 S	Forward	ggtcaaatgagcctatgggg
5418 R	Forward	ggtcaaatgagcctatgggc
5548	Reverse	CTGTTGTTAGGCTCTTCAATG

4. RESULTS

4.1. *In vitro* sensitivity assays of Italian and Turkish populations

Italian populations

Trifloxystrobin sensitivities of 55 populations (1 untreated, 9 wild type, 4 good field performance, 36 poor control by strobilurins, 1 biological orchard, 3 treated without use of strobilurin and 1 orchard with unknown field management) collected from Northern Italy between 2002 and 2011 were established. The *in vitro* sensitivity assays were concerned the conidial germination. The maximum EC_{50} and other *in vitro* results of the populations are shown in Table 7.

Assays to evaluate the effect of SHAM on spore germination showed that including SHAM in fungicide solutions did not significantly change the estimated EC_{50}. The addition of SHAM didn't increase the activity of trifloxystrobin on two resistant populations (n.150, 609). Mean EC_{50} value for both populations were >10 mg L^{-1} in germination assay with SHAM and without SHAM. Slightly influence of SHAM in combination with trifloxystrobin was detected on one sensitive population (n.156) (Table 6). Mean EC_{50} value was 0.0015 mg L^{-1} and 0.0007 mg L^{-1}, without SHAM and with SHAM, respectively.

Table 6. Results of *in vitro* sensitivity tests mixed with SHAM

	Germination test trifloxystrobin + SHAM		**Germination test trifloxystrobin**	
Isolate ID	**Relative germination (RG) mean %**	**EC_{50} mean (mg/L)**	**Relative germination (RG) mean %**	**EC_{50} mean (mg/L)**
611	59.87	>10	72.87	>10
551	70.87	>10	79.45	>10
115	0	0.0007	0	0.0017

Populations were categorized with mean EC_{50} < 0.049 mg L^{-1} and max EC_{50} < 0.065 mg L^{-1} as sensitive and EC_{50} max > 0.169 mg L^{-1} as resistant according with Fiaccadori *et al*., 2005.

Trifloxystrobin was highly active against to the wild type populations and untreated populations. Wild type populations showed a very wide range of sensitivity and had highly low EC_{50} values. Wild type populations can be considered very sensitive to trifloxystrobin. They showed maximum EC_{50} values ranging from 0.000079 to 0.03 mg L^{-1} and the mean RG values ranged from 0 to 9.2%. There were no germinated spores at maximum concentration (2mg L^{-1}) in six out of ten populations.

As regards four populations collected in orchards with good field performance of strobilurins showed a low maximum EC_{50} ranging from 0.0037 to 0.076 mg L^{-1} and RG ranging from 0 to14.6 %. They showed often slightly low sensitivity respect to wild types and they are considered sensitive according to sensitivity classification mentioned above.

Populations sampled mainly in orchards with poor control by strobilurins, showed a very low sensitiviy to trifloxystrobin *in vitro*. The results demonstrated that they were found to have higher EC_{50} values towards trifloxytrobin than wild type populations. The maximum EC_{50} values ranged from 0.17 to >10 mg L^{-1} and RG values of resistant populations were almost always higher than 23.3%. In one case (n.225), RG was observed with a lower value (11.4%) (Table 7). According to the results, they can be considered resistant.

The other five populations presented one from a biological orchard (n.130), three from treated without use of strobilurin (201, 600, BA3) in the year of assesment and one from an orchard with unknown field management were defined as resistant. Their maximum EC_{50} values ranged from 0.19 to >10 mg L^{-1} and the mean RG values ranged from 14.6 to 71.8%. So they can be considered as resistant.

Table 7. Results of *in vitro* sensitivity tests on conidial germination on *Venturia inaequalis* populations with different scab management from Italy

Strobilurin Management[a]	**Population ID**	**EC $_{50}$ mean (mg/L)**	**EC $_{50}$ max (mg/L)**	**Relative germination mean (RG) %**	**Sensitivity classification**[b]
WT and UNT	62	0.000051	0.000079	0	S
	122	0.0007	0.0009	0	
	426	0.0004	0.0007	0	
	12III	0.0001	0.0013	0	
	158	0.001	0.0018	0	
	115	0.0017	0.0044	0	
	228 I	0.0077	0.01	0.6	
	226	0.0077	0.012	2.4	
	1202	0.009	0.01	0.8	
	136	0.013	0.03	9.2	
GC	202	0.003	0.0037	0	S
	96	0.023	0.03	6.1	
	156	0.012	0.034	14.6	
	427[d]	0.015	0.076[d]	12.5	
PC	319	0.097	0.17	42	R
	408	0.056	0.17	24.5	
	229	0.09	0.18	29	
	225	0.23	0.25	11.4	
	714	0.25	0.3	35.54	
	144	0.3	0.38	35,03	
	302	0.092	0.85	23,3	
	533	0.086	1.24	39.76	
	523	0.38	1.46	44.5	
	161	0.098	2.56	31.4	
	602	1.1	5.4	50.3	
	412	4.2	5.4	55.5	
	102	4.2	>10	51.6	
	88	7.9	> 10	46.5	
	307	1.7	>10	49.3	
	411	2.7	>10	53.8	
	138	1.49	>10	56.0	
	507	7.35	>10	56.5	
	508	3.68	>10	58.14	
	707	6.86	>10	65.3	
	504	>10	>10	66.45	
	535	>10	>10	66.71	
	616	>10	>10	67.02	
	605	>10	>10	68.17	
	512	>10	>10	68.19	
	611	>10	>10	72.87	

Strobilurin Management[a]	Population ID	EC $_{50}$ mean (mg/L)	EC $_{50}$ max (mg/L)	Relative germination mean (RG) %	Sensitivity classification[b]
PC	708	>10	>10	74.56	R
	506	3.41	>10	76.29	
	503	>10	>10	76.43	
	550	>10	>10	77.6	
	551	>10	>10	79.45	
	546	>10	>10	84.9	
	543	>10	>10	87.97	
	549	>10	>10	88.0	
	87	>10	>10	97.3	
	706	ND[c]	ND[c]	ND[c]	ND[c]
NOS	201	0.12	0.19	14.6	R
	600	0.21	2.22	34.33	
	BA3	>10	>10	68.8	
BIO	130	0.16	2.6	23.9	R
ND	612	>10	>10	71.8	R

[a] Wt: wild-type; UNT: untreated, NOS: no strobilurins used; GC: good control by strobilurins; PC: poor control by strobilurins; ND: Informations not available

[b] S= sensitive EC_{50} max < 0.065mg/L; R= resistant EC_{50} max > 0.169 mg/L

[c] ND; no data because of less conidia germination in *in vitro* test

[d] EC $_{50}$ max is slightly superior than higher value for sensitive population

Turkish populations

A total of 9 populations from western and southern Turkey were tested with the *in vitro* test. The maximum EC_{50} values ranged from 0.0008 to 0.05 mg L^{-1} and the mean RG values ranged from 0 to 8.65% (Table 8). They can be considered very sensitive to trifloxystrobin.

The data couldn't obtain for populations collected in orchards with poor field performance of strobilurins because of the less germination or non vital spores in conidial germination tests.

Table 8. Results of *in vitro* sensitivity tests on conidial germination on *Venturia inaequalis* populations with different origins from Turkey

Origin of populations	**Population ID**	**EC_{50} mean (mg/L)**	**EC_{50} max (mg/L)**	**Relative germination mean (RG) %**	**Sensitiviy classification**
Wild type and untreated	700	0.00001	0.0008	0	S
	709	0.009	0.01	0	
	711	0.004	0.01	0.35	
	713	0.015	0.015	0	
	712	0.04	0.05	8.65	
Poor control	701	ND	ND	ND	
	702				
	703				
	710				

S= sensitive EC_{50} max < 0.065mg/L; R= resistant EC_{50} max > 0.169 mg/L
ND; no data because of less conidia germination or non vital spores *in vitro* test
* Log EC_{50} mean

4.2 Results of *In vitro* sensitivity assays of monoconidial isolates

A total of 60 monoconidal isolates; 24 monoconidial spores from 2 wild type populations (n.62 and n.1202), 21 monoconidial spores from populations sampled in orchard well controlled by strobilurins (n.202 and n.156), 15 monoconidial spores from populations sampled from orchards where strobilurins showed control failures (n.102 and n.503) were tested for trifloxystrobin sensitivity tests in laboratory. Conidial germination and mycelial growth inhibition on growth medium with trifloxystrobin were assessed and sensitivity parameters (EC_{50} values) were determined using probit analysis and relative germination was calculated.

It was observed that the addition of SHAM didn't increase the activity of trifloxystrobin on sensitive isolates (Table 9).

Table 9. Results of *in vitro* sensitivity tests mixed with SHAM

	Germination test trifloxystrobin + SHAM		Germination test trifloxystrobin	
Isolate ID	**Relative germination (RG) mean %**	**EC_{50} mean (mg/L)**	**Relative germination (RG) mean %**	**EC_{50} mean (mg/L)**
1202-56	3.6	0.02	11.17	0.02
1202-75	17.3	0.02	16.8	0.06
1202-100	18.95	0.002	24.79	0.004

Examing the sensitivity results of wild-types, 12 monoconidial isolates of population (n.62) showed a high sensitivity. From the results shown in Table 10, mean EC_{50} values ranged from 0.0003 to 0.003 mg L^{-1} (mean=0.001 mg L^{-1}). RG of 9 monoconidial isolates was < 1% and the other three (62-36, 62-38, 62-39) had RG from 1.78 to 6.05%. In mycelial growth assay, mean EC_{50} values ranged from 0.0002 to 0.01 mg L^{-1} (mean=0.0037 mg L^{-1}) and the relative growth ranged from 0 to 9.09%. Mean EC_{50} values of all monoconidal isolates from mycelial growth assays were found slightly higher than mean EC_{50} values of germination assays.

Two monoconidial isolates (62-4, 62-38) were inoculated on seedlings and conidial germination test with obtained conidia from infected leaves results showed EC_{50} 0.00003 mg L^{-1} and RG was 0% for both, while slightly low sensitivity found in germination assay after cellophane (EC_{50} 0.001 mg L^{-1} and 0.0008 mg L^{-1} with 0% and 6.05% RG, respectively).

Table 10. Results of *in vitro* sensitivity tests on conidial germination and mycelial growth on monoconidial isolates of wild type population (62)

		conidial germination		mycelial growth		conidial germination from inoculated leaves		EC_{50} Population (mg/L)
Population ID and origin	**Isolate ID**	**Relative germination mean %**	**EC_{50} mean (mg/L)**	**Relative growth Mean %**	**EC_{50} mean (mg/L)**	**Relative germination mean %**	**EC_{50} mean (mg/L)**	
62 Wild type	**62-1**	0.9	0.003	0	0.001	nd[1]	nd[1]	**0.00005**
	62-4	0[a]	0.001[a]	5.5	0.004	0	0.00003	
	62-8	0.5	0.001	9.52	0.01	-	-	
	62-10	0	0.001	0	0.002	-	-	
	62-15	0.5	0.002	0	0.005	-	-	
	62-26	0.4	0.0007	0	0.03	-	-	
	62-30	0.9	0.001	0	0.002	-	-	
	62-33	0	0.001	nd	nd	-	-	
	62-35	0.8	0.0008	0	0.0002	-	-	
	62-36	1.78	0.0015	0	0.007	-	-	
	62-38	6.05[a]	0.0008[a]	9.09	0.008	0	0.00003	
	62-39	2.43	0.0003	9.09	0.006	-	-	
Mean value		**1.18**	**0.001***	**3.01**	**0.0037***			
Mean value[a]		**2.01**	**0.0009**			**0**	**0.00003**	

* Geometric mean (Log EC_{50} mean)
[1] nd= no data

For monoconidial isolates of the other wild-type population (n.1202), mean EC_{50} values ranged from 0.004 to 0.11 mg L^{-1} (mean=0.02 mg L^{-1}) and RG showed ranging from 0 to 24.79% (Table 11). In mycelial growth assay, they showed mean EC_{50} ranging from 0.001 to 0.1 mg L^{-1} (mean=0.01 mg $L^{-}1$) and relative growth was <23%. The mean EC_{50} values of all monoconidal isolates tested in germination and mycelial growth tests were higher than that of correspondings populations (EC_{50} 0.009 mg L^{-1}), nevertheless, they were found in a range of high sensitivity. The four isolates tested after cellophane methodology were also evaluated the sensitivity after inoculation on seedlings and showed lower EC_{50} values respect to those results after cellophane methodology.

Table 11. Results of *in vitro* sensitivity tests on conidial germination and mycelial growth on monoconidial isolates of wild type population (1202)

		conidial germination		mycelial growth		conidial germination from inoculated leaves		EC_{50} mean Population (mg/L)
Population ID and origin	**Isolate ID**	**Relative germination mean %**	**EC_{50} mean (mg/L)**	**Relative growth Mean %**	**EC_{50} mean (mg/L)**	**Relative germination mean %**	**EC_{50} mean (mg/L)**	
1202 Wild type	**1202 - 27**	nd	nd	4.0	0.09	-	-	0.009
	1202 - 29	9.12	0.05	10.0	0.001	-	-	
	1202 - 42	9.9	0.07	0	0.003	-	-	
	1202 - 46	8.83[a]	0.01[a]	5.0	0.08	0	0.00006	
	1202 - 47	11.71[a]	0.11[a]	3.5	0.005	0.96	0.0025	
	1202 - 56	11.17[a]	0.02[a]	10.0	0.03	6.46	0.06	
	1202 - 64	10.3	0.02	0	0.01	-	-	
	1202 - 72	3.5[a]	0.009[a]	0	0.1	0	0.00045	
	1202 - 75	16.8	0.06	4.0	0.01	-	-	
	1202 - 77	0	0.007	23.07	0.02	-	-	
	1202 - 83	0	0.009	9.09	0.004	-	-	
	1202- 100	24.79	0.004	4.1	0.009	-	-	
Mean value		**8.84**	**0.02***	**6.06**	**0.01***			
Mean value[a]		**8.80**	**0.02***			**1.85**	**0.0014***	

* Geometric mean (Log EC_{50} mean)
[1] nd= no data

8 monoconidial isolates of population (n.202) collected from orchards with good scab control by strobilurins showed mean EC_{50} values ranging from 0.015 to 0.04 mg L^{-1} (mean=0.029 mg L^{-1}) and RG showed ranging from 0.8 to 8.5% (Table 12). According to the mycelium growth assay, their sensitiviy were slightly lower than conidial germination test and mean EC_{50} values ranged from 0.01 to 0.25 mg L^{-1} (mean=0.07 mg L^{-1}). The relative growth ranged from 9.09 to 25%. The mean EC_{50} of all monoconidial isolates was slightly higher than those populations's EC_{50} (0.0037 mg L^{-1}) in both assays but they were found still high sensitive to trifloxystrobin.

Isolates n.202-62 and n.202-89 showed high sensitivity with EC_{50} values 0.0002, 0.002 mg L^{-1} and RG 0 %, 8.07 %, respectively, in conidial germination test with conidia obtained from inoculated leaves respect to conidial germination test after cellophane methodology (EC_{50} 0.02 and 0.0015 mg L^{-1}, respectively) (Table 12).

Table 12. Results of *in vitro* sensitivity tests on conidial germination and mycelial growth on monoconidial isolates of population (202) from orchards with good scab control by strobilurins

		conidial germination		**mycelial growth**		**conidial germination from inoculated leaves**		**EC_{50} Population (mg/L)**
Population ID and origin	**Isolate ID**	**Relative germination mean %**	**EC_{50} mean (mg/L)**	**Relative growth Mean %**	**EC_{50} mean (mg/L)**	**Relative germination mean %**	**EC_{50} mean (mg/L)**	
202 Orchards with good scab control by strobilurins	**202-7**	7.1	0.04	9.09	0.02	-	-	0.0037
	202-31	2.43	0.038	11.1	0.067	-	-	
	202-35	8.5	0.05	9.09	0.01	-	-	
	202-62	3.8[a]	0.02[a]	10.5	0.102	0	0.0002	
	202-66	2.06	0.016	-	-	-	-	
	202-76	0.8	0.023	11.1	0.06	-	-	
	202-77	3.83	0.03	13.65	0.01	-	-	
	202-89	3.98[a]	0.015[a]	25.0	0.25	8.07	0.002	
Mean value		**4.06**	**0.029**	**12.79**	**0.07***			
Mean alue[a]		**3.89**	**0.01**			**4.03**	**0.0006***	

* Geometric mean (Log EC_{50} mean)

In most cases, trifloxystrobin sensitivity of monoconidial isolates of population n.156 sampled in well controlled by strobilurins, was lower than sensitivity of those population (EC_{50} 0.01 mg L^{-1}) in conidal germination test. The range of the EC_{50} of monoconidial isolates was from 0.009 to 0.3 mg L^{-1} (mean=0.04) (Table 13). RG showed ranging from 1.9 to 45.47% (mean=11.85%).

Table 13. Results of *in vitro* sensitivity tests on conidial germination on monoconidial isolates of population (156) from orchards with good scab control by strobilurins

		conidial germination		mycelial growth		conidial germination from inoculated leaves		EC_{50} Population (mg/L)
Population ID and origin	**Isolate ID**	**Relative germination mean %**	**EC_{50} mean (mg/L)**	**Relative growth Mean %**	**EC_{50} mean (mg/L)**	**Relative germination mean %**	**EC_{50} mean (mg/L)**	
156 **Orchard with good scab control by strobilurins**	**156-1**	5.4	0.039	-	-	-	-	0.012
	156-2	16.50	0.067	-	-	-	-	
	156-3	6.6	0.017	-	-	-	-	
	156-4	23.3	0.033	-	-	-	-	
	156-5	5.78	0.032	-	-	-	-	
	156-6	4.21	0.017	-	-	-	-	
	156-7	14.8	0.037	-	-	-	-	
	156-8	1.9	0.009	-	-	-	-	
	156-9	45.47	0.387	-	-	-	-	
	156-12	11.7	0.015	-	-	-	-	
	156-13	3.47	0.012	-	-	-	-	
	156-16	11.1	0.019	-	-	-	-	
	156-17	3.83	0.025	-	-	-	-	
Mean value		**11.85**	**0.04***					

* Geometric mean (Log EC_{50} mean)

Mean EC_{50} values (mostly EC_{50} >10 mg L^{-1}) ranged for monoconidial isolates of population n. 102 and 503 collected from orchards where strobilurins showed control failures showed the highest EC_{50} for trifloxystrobin (Table 14 and Table 15). In conidial germination assay, all monocondial isolates of population n.102 and 5 out of the 6 monoconidial isolates of n. 503 had mean EC_{50} >10 mg L^{-1} and RG was >71 %. Only one monoconidial isolate (n.503-6) was slightly less resistant with mean EC_{50} 6.07 mg $L^{-1}$and RG 53.1%.

In mycelial growth assay, the results obtained for 4 monoconidal isolates out of 9 for n.102 and for 2 monoconidial isolates out of 6 for n.503. For monocondial isolates of n.102, mean EC_{50} ranged from 1.14 to >10 mg L^{-1} and RG was from 37.5 to 100%. For

monocondial isolates of population 503, mean EC_{50} ranged from 1.68 to >10 mg L^{-1} and RG was > 60% (mean EC_{50} 9.12 mg L^{-1}). All monoconidal isolates of the resistant bulk populations (n.102 and n.503) were strongly less sensitive to trifloxystrobin in both assays, resulting in very high EC_{50} values. Trifloxystrobin showed lower activity against resistant isolates compared to wild-types.

Table 14. Results of *in vitro* sensitivity tests on conidial germination and mycelial growth on monoconidial isolates of population (102) from orchards with poor scab control by strobilurins

		conidial germination		**mycelial growth**		**conidial germination from inoculated leaves**		**EC_{50} Population (mg/L)**
Population ID and origin	**Isolate ID**	**Relative germination mean %**	**EC_{50} mean (mg/L)**	**Relative growth Mean %**	**EC_{50} mean (mg/L)**	**Relative germination mean %**	**EC_{50} mean (mg/L)**	
102 **Orchard with control failures by strobilurins**	**102-5**	73.78	>10	72.72	1.28	-	-	>10
	102-9	84.64	>10	91.6	Nd	-	-	
	102-10	81.99[a]	>10[a]	50	2.74	62.55	>10	
	102-13	71.95[a]	>10[a]	75	>10	50.8	3.9	
	102-44	79.06	>10	37.5	1.14	-	-	
	102-55	77.47	>10	83.3	Nd	-	-	
	102-68	77.45	>10	100	Nd	-	-	
	102-1	71.3	>10	nd	Nd	-	-	
	102-3	78.1	>10	75.0	>10	-	-	
Mean value		**77.30**	**>10**	**73.14**				
Mean value[a]		**76.97**	**>10**			**56.67**		

After cellophane methodology, conidial suspension of two monoconidial isolates (n.102-10 and n.102-13) were inoculated on seedlings to test conidial germination assay with conidia obtained from inoculated leaves. Examing the data referred to EC_{50} parameters, it was observed a similar trend. They had high resistant values with mean EC_{50} 14.1 mg L^{-1} and EC_{50} 3.9 mg L^{-1} and their RG were 62.55% and 50.8%, respectively.

Table 15. Results of *in vitro* sensitivity tests on conidial germination and mycelial growth on monoconidial isolates of population (503) from orchards with poor scab control by strobilurins

		conidial germination		mycelial growth		conidial germination from inoculated leaves		
Population ID and origin	**Isolate ID**	**Relative germination Mean %**	**EC_{50} mean (mg/L)**	**Relative growth Mean %**	**EC_{50} mean (mg/L)**	**Relative germination Mean %**	**EC_{50} mean (mg/L)**	**EC_{50} Population (mg/L)**
503 Orchard with control failures by strobilurins	**503-6**	53.1	6.07	nd	Nd	-	-	>10
	503-10	89.5	>10	nd	Nd	-	-	
	503-13	71.8	>10	66.7	1.68	-	-	
	503-18	92.3	>10	60.0	>10	-	-	
	503-19	88.6	>10	nd	Nd	-	-	
	503-23	88.3	>10	nd	Nd	-	-	
Mean value		**80.6**		**63.35**				

4.3. Molecular results

Real Time analyses were performed with SybrGreen, with the primer set both for wild-type and for mutant allele to evaluate the percentage of mutated allele through Delta Ct method (Fig 14). The melting curve analyses of qPCR for all populations and monoconidial isolates showed a single peak, and no non specific products or primer-dimer formation was detected. The standard curves obtained using, mixed strains (50%S-50%R) sensitive and resistant in 10-fold serial diluition showed a good efficiency (E) with this primer sets (5548Rev+5418S), (5548Rev+5418R) for qPCR ranging from E=100.1% to E=101.9%, with a linear correlation coefficient (R^2) ranging from 0.999 to 1.000 (Fig 15 and 16). The high efficiency for each gene allowed the assumption that the genes are amplified with the same efficiency, and an average slope of – 3.298 was used in the equation.

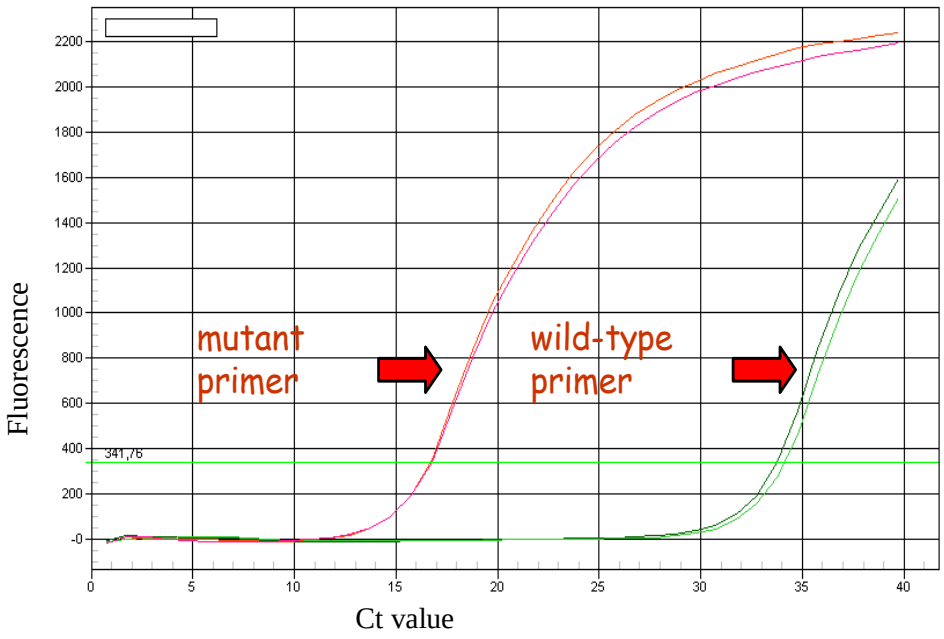
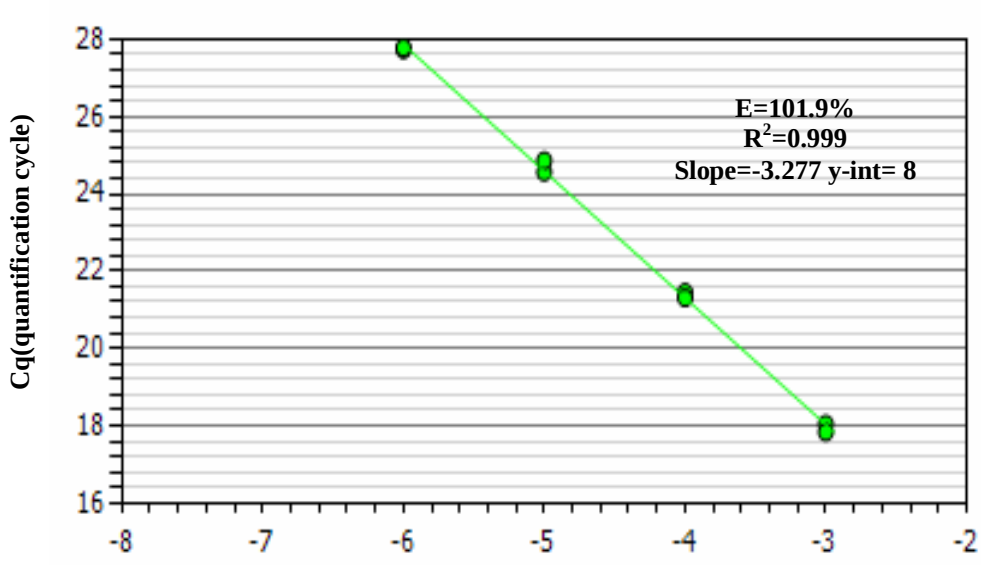


Fig.14. Example of amplification profiles of resistant population



Log of standard curve (50%S+50%R) with sensitive primers

Fig.15. Standard curves with sensitive primer

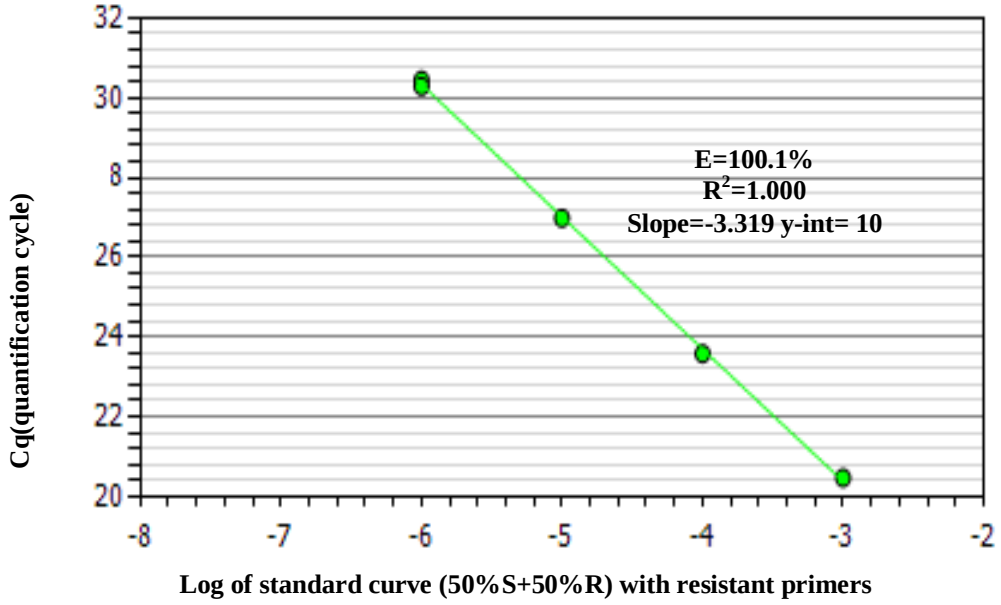


Fig.16. Standard curves with resistant primer

From 2002 to 2011, infected leaves of 64 populations were sampled in different origin from Italy and Turkey. Bulk population samples extracted from *V. inaequalis*–infected apple leaves collected in orchards and monoconidial isolates extracted from conidia to detect the mutation in cytochrome b gene causing the G143A substitution in the protein by CAPS analysis. To quantify the G143 and A143 alleles in population and monoconidial isolates, real-time allele-specific PCR assays were performed using the fluorescent dsDNA-specific dye SYBR Green I. The analyses were repeated two or three times for all populations and monoconidial isolates.

4.3.1 Molecular results of italian populations

According to qualitative analysis (CAPS PCR) results, G143A substitution was not found in 6 out of total 9 wild-type and 1 untreated populations and their frequency of mutated alleles (R-allele) was found from 0.02 to 5.5% by qPCR (Table 16). G143A

substitution and high mutated allele frequency were surprisingly found in four wild-type populations (n.426, 158, 226, 228I).

It was observed a partial digestion in CAPS analysis (a showing two fragments of 400 and 300bp), which means that sensitive and resistant strains were present both in these 4 populations. CAPS method is able to detect a percentage of mutated allele greater than about 10% (evaluated according to the methodology used by Baumler *et al.*, 2003). But a fragment of 300bp was detected lighter than fragment of 400bp for these wild type populations (Fig 17). Their R-allele frequencies between 17.55% and 21.32% were measured by qPCR.

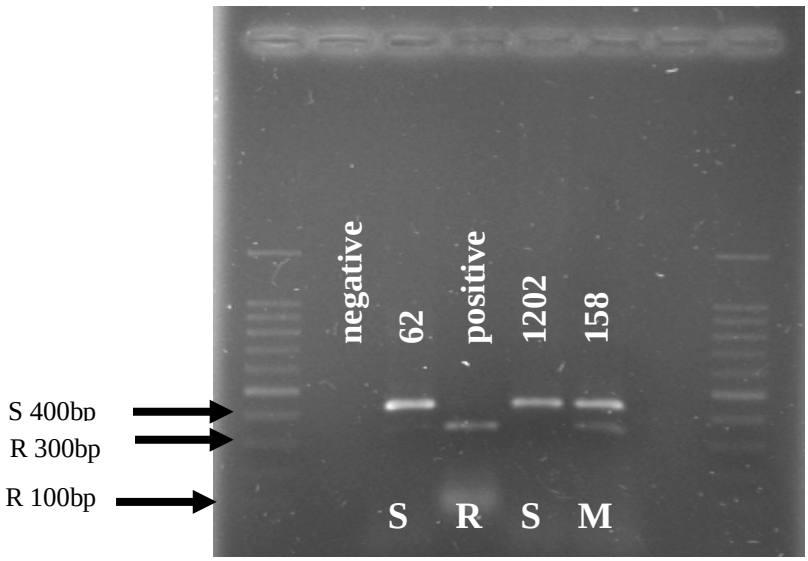


Fig.17. Digestion of PCR products with TseI enzyme

G143A substitution detected in one of the 4 populations (n.427) sampled in well controlled orchard by strobilurins by the observation of mixtures of G143 and A143 alleles. The R-allele frequency was found very high (84.5%). The G143A substitution wasn't detected in other three populations by CAPS analysis and their R-allele frequency found < 0.6% (Table 16).

In 35 populations out of 36 populations sampled from orchards where strobilurins showed control failures, G143A substitution was found. *In vitro* results couldn't obtain for population 706 but G143A substitution was detected with molecular analysis.

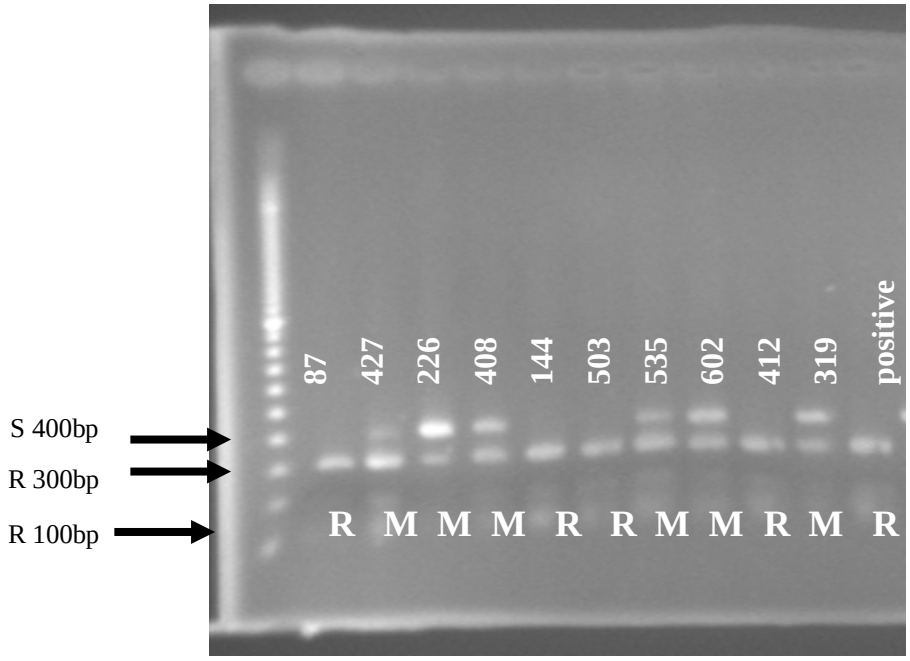


Fig.18. Digestion of PCR products with TseI enzyme

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In figure 18, TseI enzyme does not cut the wildtype 400bp cytochrome *b* fragment, but produces a 300bp fragment from isolates carrying the G143A substitution.

The mixture of sensitive and resistant strains was observed in 8 of out of 36 resistant population by CAPS and their R-allele frequencies ranged from 23.01 to 78.29 %. Only resistant strains found in 27 populations with the high R-allele frequency (>91%). G143A substitution was not detected in only one population (161) and the R-allele frequency was quite low (5.97%) (Table 16). Three populations sampled from orchards where non strobilurin used and G143A substitution was found in two of them with the R-allele frequency ranged from 54.87 to 56.77%. The other population had 0.015% R-allele frequency.

G143A substitution detected in one population sampled in biological orchard by the observation of mixtures of G143 and A143 alleles and the R-allele frequency found 61.21%.

Table 16. Molecular analysis results of Italian Populations

Population ID	Management characteristics[1]	CAPS interpretation[2]	Mean percentage of A143 allele		Population ID	Management characteristics	CAPS interpretation[2]	Mean percentage of A143 allele
62	WT	S	0.7		412	PC	R	98.61
1202	WT	S	0.5		102	PC	R	99.5
122	WT	S	0.07		88	PC	R	99.09
426	WT	M	18.93		307	PC	R	99.21
202	GC	S	0.015		411	PC	R	99.49
12III	WT	S	0.02		138	PC	R	99.54
158	WT	M	21.32		507	PC	R	99.15
115	UNT	S	2.28		508	PC	R	98.51
228 I	WT	M	21.11		707	PC	M	78.29
226	WT	M	17.55		504	PC	R	99.4
96	GC	S	0.55		535	PC	M	72.73
136	WT	S	5.5		616	PC	R	99.26
156	GC	S	0.02		605	PC	R	93.35
427	GC	M	84.5		512	PC	R	99.24
319	PC	M	31.01		BA3	NOS	M	54.87
408	PC	M	68.2		612	ND	M	89.63
229	PC	R	91.68		708	PC	R	95.9
201	NOS	S	0.015		611	PC	R	98.29
225	PC	M	32.8		506	PC	R	91.74
144	PC	R	99.27		503	PC	R	99.54
714	PC	R	96.88		550	PC	R	99.32
302	PC	M	76.1		551	PC	R	95.2
533	PC	M	23.01		546	PC	R	99.29
523	PC	R	99.51		543	PC	R	99.21
161	PC	S	5.97		549	PC	R	97.8
600	NOS	M	56.77		87	PC	R	99.07
130	BIO	M	61.21		706	PC	R	98.59
602	PC	M	56.79					

[1]Wt: wild-type; UNT: untreated, NOS: no strobilurins used; GC: good control by strobilurins; PC: poor control by strobilurins

[2]S= Sensitive, R= Resistant, M= mixed

4.3.2 Molecular results of Turkish populations

The G143A substitution was not found in any of wild type and untreated Turkish populations by CAPS analysis and the frequency of mutated allele was found <3.61%.

Five populations sampled from orchards where strobilurins showed control failures, a mutation resulting in the replacement of a glycine by an alanine residue at codon 143 (G143A) in the mitochondrial cytochrome b gene was found (Fig 19).

The mixture of sensitive and resistant strains was observed in three populations by CAPS and their R-allele frequencies ranged from 46.62% to 83.36%. Much higher R-allele frequency (>95%) found in other two populations. *In vitro* results couldn't obtain for population 701, 702, 703 and 710 because of the less germination conidia but resistance to strobilurin was detected with not vital conidia by molecular analysis (Table 17).

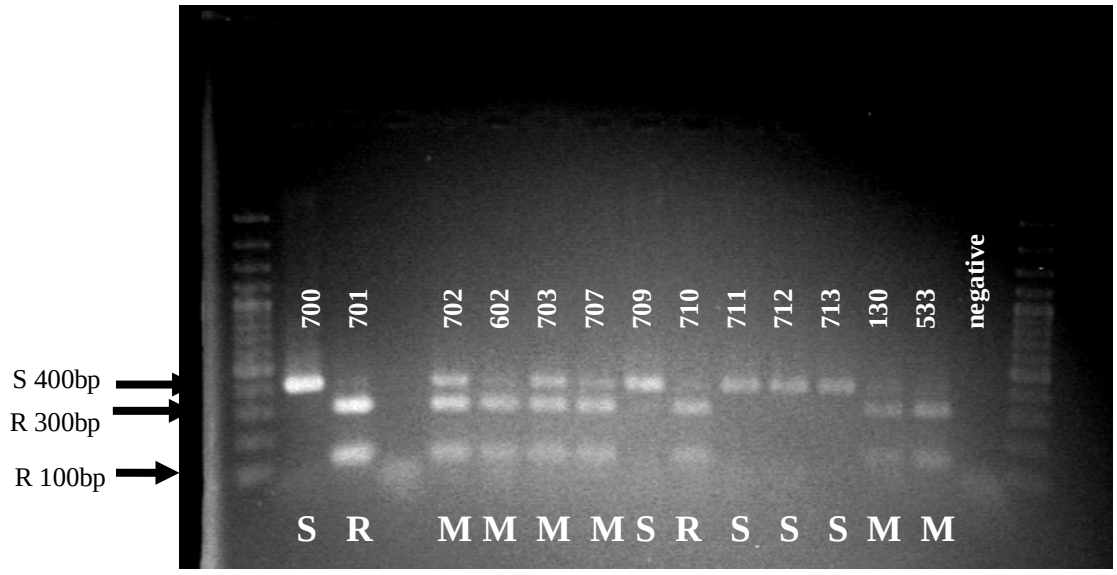

Fig. 19. Digestion of PCR products with TseI enzyme

Table 17. Molecular analysis results of Turkish populations

Population ID	Management characteristics	CAPS interpretation	Mean percentage of A143 allele
700	WT	S	0.1
709	UNT	S	3.61
713	UNT	S	0.25
711	UNT	S	0.3
712	UNT	S	0.6
707	PC	M	78.29
701	PC	R	99.76
702	PC	M	46.62
703	PC	M	83.36
710	PC	R	95.99

4.3.3. Molecular results of monoconidal isolates

A total of 24 monoconidial isolates from 2 wild type populations (62 and 1202), 21 monoconidial isolates from populations sampled in orchard well controlled by strobilurins (202 and 156), 15 monoconidial isolates from populations sampled from orchards where strobilurins showed control failures (102 and 503) were tested. To determine the frequency of mutant G143A allele in samples, tests were conducted using spore suspensions utilizing Chelex 100. Some monoconidal isolates (156-2, 156-3, 156-4, 156-6, 156-7, 156-9 and 503-18) were extracted also by a CTAB - based method to compare the effectiveness of Chelex method. DNA of monoconidial isolates obtained using the two different extraction methods were identical for all samples tested (Table 19 and 20).

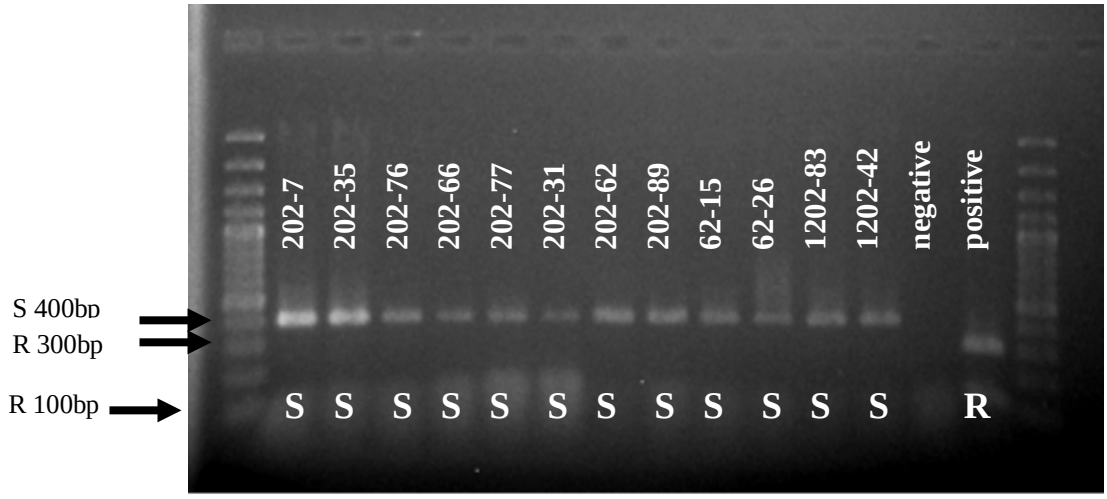

Fig. 20. Digestion of PCR products of sensitive monoconidial isolates with TseI enzyme

The G143A substitution was not found in any of monoconidial isolates of wild type and populations sampled in orchard well controlled by CAPS PCR (Fig.20). The percentage of resistant alleles in monoconidial *V. inaequalis* isolates of two wild type populations ranged from 0.01 to 1.66% (Table 18).

Percentage of R-allele for monoconidal isolates of populations with good scab control found between 0.04 to 1.04% (Table 19).

Table 18. Molecular results of monoconidial isolates obtained from wild type populations

Monoconidial Isolates No	CAPS interpretation	Mean percentage of A143 allele		Monoconidial Isolates No	CAPS interpretation	Mean percentage of A143 allele
62-1	S	0.25		1202-27	S	0.28
62-4		0.35		1202-29		0.54
62-8		0.52		1202-42		0.01
62-10		0.25		1202-46		0.15
62-15		0.72		1202-47		0.59
62-26		0.57		1202-56		0.03
62-30		0.23		1202-64		0.96
62-33		0.23		1202-72		1.66
62-35		0.13		1202-75		1.58
62-36		0.3		1202-77		0.19
62-38		0.2		1202-83		0.01
62-39		0.3		1202-100		0.03

Table 19. Molecular results of monoconidial isolates obtained from populations sampled in well controlled orchard by strobilurins

Monoconidial Isolates No	CAPS interpretation	Mean percentage of A143 allele	Monoconidial Isolates No	CAPS interpretation	Mean percentage of A143 allele
156-1	S	0.11	202-7	S	0.06
156-2		0.32[a] / 0.51[b]	202-31		0.16
156-3		0.31[a] / 0.04[b]	202-35		0.04
156-4		0.25[a] / 0.16[b]	202-62		0.3
156-5		0.18	202-66		0.04
156-6		0.13[a] / 0.17[b]	202-76		0.04
156-7		0.08[a] / 0.12[b]	202-77		0.16
156-8		0.10	202-89		0.04
156-9		0.10[a] / 0.07[b]			
156-12		0.8			
156-13		1.04			
156-16		0.6			
156-17		1.09			

[a] Results obtained from extraction by Chelex 100.
[b]Results obtained from extraction by a CTAB - based method

Mixtures of G143 (S) and A143 (R) - alleles were never found in resistant monoconidal *V. inaequalis* isolates of resistant populations by CAPS PCR and their high R-allele frequencies were detected between 98.43 to 99.9% (Table 20).

Table 20. Molecular results of monoconidial isolates obtained from populations sampled where strobilurins showed control failures

Monoconidial Isolates No	CAPS interpretation	Mean percentage of A143 allele	Monoconidial Isolates No	CAPS interpretation	Mean percentage of A143 allele
102-5	R	99.7	503-6	R	98.81
102-9		99.7	503-10		99.1
102-10		99.7	503-13		98.46
102-13		99.9	503-18		98.43[a] / 96.59[b]
102-44		99.5	503-19		99.26
102-55		99.6	503-23		99.21
102-68		99.5			
102-1		98.92			
102-3		99.16			

[a] Results obtained from extraction by Chelex 100
[b]Results obtained from extraction by a CTAB - based method

4.3.4 Correlation between qPCR assays and biological tests

Total 64 *V. inaequalis* populations and 60 monoconidal isolates were tested in order to compare the conventional method with the qPCR assay for quantifying mutated allele. The results couldn't obtain *in vitro* for five population and one monoconidial isolate because of the very low germination or not vital conidia. The frequency of mutated allele of all populations and monoconidial isolates was detected by qPCR. The percentage of relative germination and percentage of mutated allele of total 59 bulk populations and 59 monoconidal isolates were correlated.

V. inaequalis populations

All results from biological and molecular assays are shown in Table 21. Populations are classified as sensitive or resistant according to EC_{50} values (Column A) obtained from *in vitro* sensitivity tests. It was observed that the populations classified as sensitive (EC_{50} max ranged from 0.000079 to 0.076 mg L^{-1}) with RG <14.6 presented the percentages of mutated alleles from 0.015 to 21.32% in 18 cases, while in only one population sampled from well control orchard by strobilurin had high R-allele frequency (84.5%) (Table 21). With referring to wild-type populations, when the relative germination is 0 %, the mutated alleles were generally ranged from 0% to 3.7%, only in three cases the R-allele frequency is reaching 21.32% (n.426, 158, 228). Totally in four wild-type populations, G143A was present an higher frequency (from 17.55 to 21.32%) than the other wild types.

Starting from n.319, according to the fungicide sensitivity *in vitro* test were defined as resistant and showed progressively high levels of EC_{50} max value (from 0.17 to >10 mg L^{-1}) and RG (from 11.4 to 97.3 %) in 40 populations of *V. inaequalis.* They were associated with the presence of G143A substitution with high R-alleles frequency (from 23.01 to

99.54%) for 38 out of 40 populations. Only two cases were out of this range and, in this case, the R-allele frequency was much lower (0.015 and 5.97%).

Table 21. *In vitro* and molecular test results of the 64 *V. inaequalis* populations from Italy and Turkey

	***In vitro* assay results**				**Molecular analysis results**	
		A	**B**	**C**	**D**	**E**
Population n°	**Management characteristics [1]**	**EC_{50} max (mg/L)**	**Sensitivity classification [2]**	**Mean percentage of relative germination %**	**Mean percentage of A143 allele**	**CAPS interpretation**
62	WT	0.000079	S	0	0.7	S
426	WT	0.0007	S	0	18.93	M
700	WT	0.0008	S	0	0.1	S
122	WT	0.0009	S	0	0.07	S
12-3	WT	0.0013	S	0	0.02	S
158	WT	0.0018	S	0	21.32	M
202	GC	0.0037	S	0	0.015	S
115	UNT	0.0044	S	0	2.28	S
1202	WT	0.01	S	0.8	0.5	S
709	UNT	0.01	S	0	3.61	S
713	UNT	0.015	S	0	0.25	S
711	UNT	0.01	S	0.35	0.3	S
228 I	WT	0.01	S	0.6	21.11	M
226	WT	0.012	S	2.4	17.55	M
96	GC	0.03	S	6.1	0.55	S
136	WT	0.03	S	9.2	5.5	S
156	GC	0.034	S	14.6	0.02	S
712	UNT	0.05	S	8.65	0.6	S
427	GC	0.076	S	12.5	84.5	M
319	PC	0.17	R	42.0	31.01	M
408	PC	0.17	R	24.5	68.2	M
229	PC	0.18	R	29.0	91.68	R
201	NOS	0.19	R	14.6	0.015	S
225	PC	0.25	R	11.4	32.8	M
144	PC	0.38	R	35.03	99.27	R
714	PC	0.3	R	35.54	96.88	R
302	PC	0.85	R	23.3	76.1	M
533	PC	1.24	R	39.76	23.01	M
523	PC	1.46	R	44.5	99.51	R
161	PC	2.56	R	31.4	5.97	S
600	NOS	2,22	R	34.33	56.77	M
130	BIO	2.6	R	23.9	61.21	M
602	PC	5.4	R	50.3	56.79	M
412	PC	5.4	R	55.5	98.61	M
102	PC	>10	R	51.6	99.5	R
88	PC	> 10	R	46.5	99.09	R
307	PC	>10	R	49.3	99.21	R
411	PC	>10	R	53.8	99.49	R
138	PC	>10	R	56.0	99.54	R

	In vitro assay results				Molecular analysis results	
		A	B	C	D	E
Population n°	**Management characteristics [1]**	**EC_{50} max (mg/L)**	**Sensitivity classification [2]**	**Mean percentage of relative germination %**	**Mean percentage of A143 allele**	**CAPS interpretation**
507	PC	>10	R	56.5	99.15	R
508	PC	>10	R	58.14	98.51	R
707	PC	>10	R	65.3	78.29	M
504	PC	>10	R	66.45	99.4	R
535	PC	>10	R	66.71	72.73	M
616	PC	>10	R	67.02	99.26	R
605	PC	>10	R	68.17	93.35	R
512	PC	>10	R	68.19	99.24	R
BA3	NOS	>10	R	68.8	54.87	M
612	ND	>10	R	71,8	89.63	M
708	PC	>10	R	74.56	95.9	R
611	PC	>10	R	72.87	98.29	R
506	PC	>10	R	76.29	91.74	R
503	PC	>10	R	76.43	99.54	R
550	PC	>10	R	77.6	99.32	R
551	PC	>10	R	79.45	95.2	R
546	PC	>10	R	84.9	99.29	R
543	PC	>10	R	87.97	99.21	R
549	PC	>10	R	88.0	97.8	R
87	PC	>10	R	97.3	99.07	R
706	PC	ND[3]	ND	ND	98.59	R
701	PC	ND	ND	ND	99.76	R
702	PC	ND	ND	ND	46.62	M
703	PC	ND	ND	ND	83.36	M
710	PC	ND	ND	ND	95.99	R

[1] Wt: wild-type; UNT: untreated, NOS: no strobilurins used; GC: good control by strobilurins; PC: poor control by strobilurins; ND: Informations not available

[2] S= sensitive EC_{50} max < 0.065mg/L; R= resistant EC_{50} max > 0.169 mg/L

[3] ND; no data because of less conidia germination in *in vitro* test

Five populations collected from orchards with poor control by strobilurin showed high R-allele frequency (> 46.62%) by qPCR, while the data couldn't obtain by *in vitro*. The correlation between relative germination (Column C) and percentage of mutated alleles (Column D) is significant (P:0.05) with a strong relationship between the two parameters as evidenced by "correlation coefficient" (0.84), while the R-squared is not very high. The results of the study showed that the correlation between % of mutated allele and relative germination is $R^2 = 0.70$ (Fig 21).

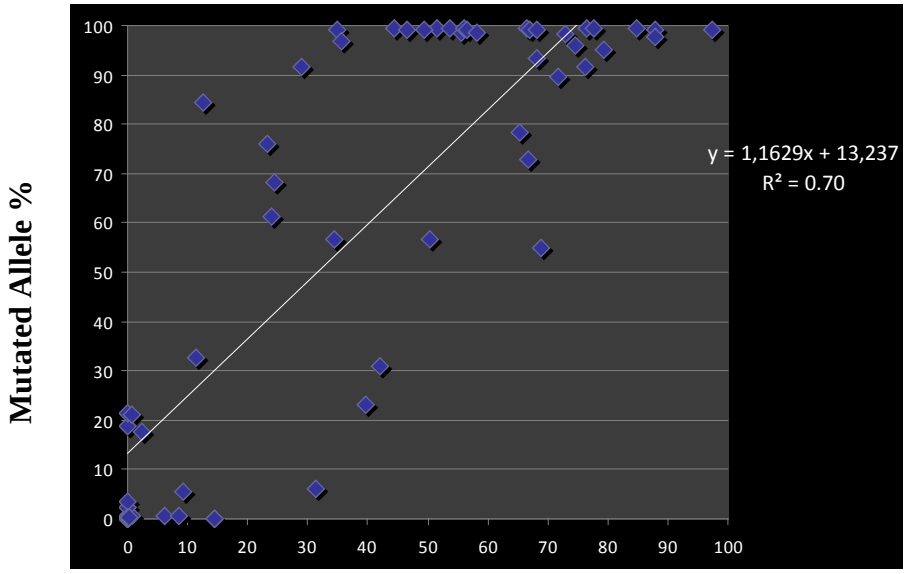

Fig. 21.Correlation between % of mutated allele and % of germinated conidia at 2mg/L of trifloxystrobin relative to the untreated control on population of *V. inaequalis*

Monoconidial isolates

As shown in Column A, all trifloxystrobin-resistant monoconidial isolates tested, with RG > 53.1% (EC_{50} > 6.07 mg L^{-1}) presented A143 cytochrome *b* alleles detected by CAPS PCR. Examing the frequency of mutated alleles, they had high R-allele frequencies between 98.43 to 99.9 % (Table 22).

Only S-alleles (G143) detected by CAPS PCR were found in all sensitive monoconidal isolates with low EC_{50} values. In almost all cases, monoconidal isolates from wild type and good control had relative germination values ranged from 0 to 11.17% while mutated allele frequency was detected lower than 1.66%. It was found that the relative germination was ranged from 11.17 to 24.79% (EC_{50} value < 0.1 mg L^{-1}) in only four single spore isolates of wild type population (n.1202) with low R-allele frequency (between 0 to 1.58%) and in only one single spore isolate from populations collected in well-controlled orchard (n.156-9), RG was found high (45.47%) (EC_{50} value 0.3 mg L^{-1}) with a very low mutated allele frequency (0.1%).

Table 22. *In vitro* and molecular test results of the monoconidal isolates of *V. inaequalis* population with differen scab management

	***In vitro* assay results**				**Molecular analysis results**	
		A	**B**	**C**	**D**	**E**
Monocondial isolates n°	**Management characteristics [1]**	**EC_{50} (mg/L)**	**Sensitivity classification [2]**	**Mean percentage of relative germination %**	**Mean percentage of A143 allele**	**CAPS interpretation**
62-1	WT	0.003	S	0.9	0.25	S
62-4		0.001		0	0.35	
62-8		0.001		0.5	0.52	
62-10		0.001		0	0.25	
62-15		0.002		0.5	0.72	
62-26		0.0007		0.4	0.57	
62-30		0.001		0.9	0.23	
62-33		0.001		0	0.23	
62-35		0.0008		0.8	0.13	
62-36		0.0015		1.78	0.3	
62-38		0.0008		6.05	0.2	
62-39		0.0003		2.43	0.3	
1202 - 27	WT	Nd[3]	S	nd	0.28	S
1202 - 29		0.05		9.12	0.54	
1202 - 42		0.07		9.9	0.01	
1202 - 46		0.01		8.83	0.15	
1202 - 47		0.11		11.71	0.59	
1202 - 56		0.02		11.17	0.03	
1202 - 64		0.02		10.3	0.96	
1202 - 72		0.009		3.5	1.66	
1202 - 75		0.06		16.8	1.58	
1202 - 77		0.007		0	0.19	
1202 - 83		0.009		0	0.01	
1202- 100		0.004		24.79	0.03	
202-7	GC	0.04	S	7.1	0.06	S
202-31		0.038		2.43	0.16	
202-35		0.05		8.5	0.04	
202-62		0.02		3.8	0.3	
202-66		0.016		2.06	0.04	
202-76		0.023		0.8	0.04	
202-77		0.03		3.83	0.16	
202-89		0.015		3.98	0.04	
156-1	GC	0.039	S	5.4	0.11	S
156-2		0.067		16.5	0.32	
156-3		0.017		6.6	0.31	
156-4		0.033		23.3	0.25	
156-5		0.032		5.78	0.18	
156-6		0.017		4.21	0.13	
156-7		0.037		14.8	0.08	
156-8		0.009		1.9	0.1	
156-9		0.387		45.47	0.1	
156-12		0.015		11.7	0.8	
156-17		0.025		3.83	1.09	

	In vitro assay results				Molecular analysis results	
		A	B	C	D	E
Monocondial isolates n°	**Management characteristics**	**EC_{50} (mg/L)**	**Sensitivity classification**	**Mean percentage of relative germination %**	**Mean percentage of A143 allele**	**CAPS interpretation**
102-5	PC	>10	R	73.78	99.7	R
102-9		>10		84.64	99.7	
102-10		>10		81.99	99.7	
102-13		>10		71.95	99.9	
102-44		>10		79.06	99.5	
102-55		>10		77.47	99.6	
102-68		>10		77.45	99.5	
102-1		>10		71.3	98.92	
102-3		>10		78.1	99.16	
503-6	PC	6.07	R	53.1	98.81	R
503-10		>10		89.5	99.1	
503-13		>10		71.8	98.46	
503-18		>10		92.3	98.43	
503-19		>10		88.6	99.26	
503-23		>10		88.3	99.21	

[1] Wt: wild-type; UNT: untreated, NOS: no strobilurins used; GC: good control by strobilurins; PC: poor control by strobilurins; ND: Informations not available

[2] S= sensitive EC_{50} max < 0.065mg/L; R= resistant EC_{50} max > 0.169 mg/L

[3] ND; no data because of less conidia germination in *in vitro* test

The correlation between relative germination and percentage of mutated alleles is highly significant (P:0.001) with a strong relationship between the two parameters as evidenced by "correlation coefficient" (0.96) and the R-squared is very high (R^2 = 0.92) (Fig. 22).

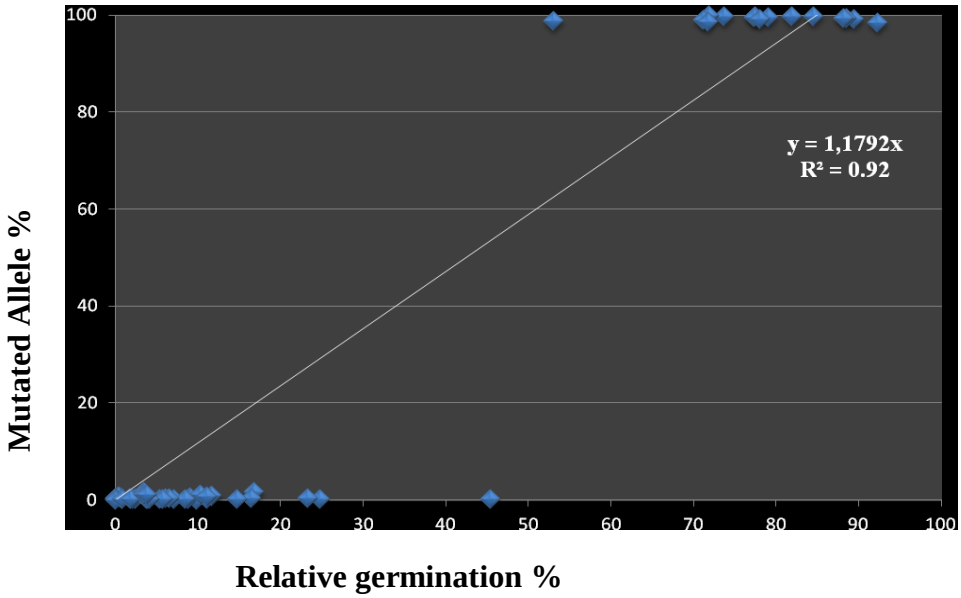

Fig. 22. Correlation between % of mutated allele and % of germinated conidia at 2mg/L of trifloxystrobin relative to the untreated control

5. Discussion

Venturia inaequalis is the most economically important fungal pathogen of apple in Italy and Turkey. Protectant and systemic fungicides have been developed, and many of these have been used against *V. inaequalis*. This fungus however, has developed resistance to several major fungicide groups and continues to be a problem in apple production. Belonging to the group of quinone outside inhibitors (QoI), were highly effective against a wide range of fungal pathogens. They are widely used for selective controlling of apple scab in orchards. However, the development of resistance mechanisms in pathogen populations was observed as a consequence of extensive application of strobilurins. G143A substitution in cytochrome b, suggested to be the major mechanism of QoI resistance, was described for a number of species of phytopathogenic fungi including *V. inaequalis* populations. This SNP was also detected in cytochrome b gene sequence, in QoI-resistant isolates in Italy (Fiaccadori *et al*., 2005; 2011).

Up to now, all screenings for resistant forms of *V. inaequalis* in the orchards in Turkey were conducted on the basis of *in vitro* assays. According to our knowledge, this study represents the first report on the detection and quantification of G143A mutation level in *V. inaequalis* fungus using qPCR assay in Turkey.

When the molecular mechanisms of resistance are known and particularly when the underlying DNA polymorphisms (single-nucleotide polymorphism [SNPs], deletions, or insertions) have been defined, various molecular methods can be used to monitor antimicrobial resistance (Fraaije, *et al*., 2002; Ma, *et al*., 2004; 2005). The principle methods for quantifying resistance are based on real-time PCR technology.

The allele-specific qPCR assay was successfully applied for the detection of QoI-resistant alleles in response to strobilurin applications (Fraaije *et a*l. 2002; Collina *et al*. 2005; Kianianmomeni *et al*., 2007; Michalecka, *et al*., 2011; Nanni, *et al*., 2011).

In this study, an allele-specific qPCR with primer sets designed was successfully developed to quantitatively determine the frequency of QoI-resistant allele 143A in populations of *V. inaequalis*. It was noticed that qPCR detects the frequency of strobilurin-resistant allele under the 10% detection limit of the CAPS method. The CAPS method well recognized the highly sensitive and resistant populations while it did not discriminate those characterised by intermediate frequencies of mutated allele. The qualitative evaluation by the CAPS PCR is certainly less useful because of its poor ability to show differences among *V. inaequalis* populations, that are often characterised by intermediate frequencies of mutated allele.

The main goal of this research was to correlate the molecular test results with *in vitro* test results, therefore percentage of mutated alleles must be compared with an *in vitro* parameter. Our previous study (Nanni *et al.*, 2011), reported as a result of sensitivity evaluation of populations, among the considered biological parameters (relative germination, % of germination at the highest concentration, EC_{50}), relative germination (RG) gave the best correlation with the percentage of mutated alleles (frequency of G143A substitution) detected by qPCR. The "EC_{50}" value showed the lowest correlation with qPCR data because it has a limited range of values (0-2 mg L^{-1}) that is not able to assess the higher levels of resistance. Moreover we considered that the "% of conidia germinated at max concentration" had a limited validity because it presented an inferior level of correlation with % of mutated alleles. Therefore, it was decided to correlate the RG values with the percentage of mutated alleles.

The results in this research have shown that the conidial germination assays demonstrated differences in sensitivity to strobilurins among populations of different origin in Italy and Turkey.

In sensitive populations, the relative germination (RG) detected within wild-type and well-controlled populations was lower than < 14.6% (maximum EC_{50} < 0.07 mg L^{-1}), while the relative germination is 0 %, R-allele frequency was generally lower than 3.7 %.

It was noticed, in some cases, high reduced *in vitro* sensitivity with low EC_{50} and low relative germination corresponded to moderately high detection of R-allele frequency for four wild type (n.426, 158, 226, 228) and one well-controlled (n. 427) populations. Detected high R-allele in wild type poopulation may be explained by the natural flow of spores from strobilurin-treated orchards to wild-type, which may travel up to 15 kilometres (Aylor *et al*., 1992). The resistant individuals can reproduce and spread over the sensitive population, this can lead to increase the resistant isolates. This indicates, as suggested by Fraaije *e*t *al*., (2005), that isolates with A143 alleles might play an important role in long-distance dispersal of QoI-resistant genotypes. Furthermore, other important biological factors, such as the tendency of fungal species to spontaneous mutations, also affect the development and spread of resistance. Therefore, strains containing mutation conferring resistance might emerge independently in each type of orchard. Subsequent fungicide application conduce an increase in resistant individuals (Michalecka *et al*., 2011). Moreover, Fungicide Resistance Action Committee (FRAC) has also shown that it is possible to detect G143A mutations in fungal populations never exposed to QoI fungicides. It would thus appear that the mutation is naturally occurring in fungal populations, albeit at low frequencies. It can also be found, again at low, but variable, frequencies, in situations where the use of QoI based products is giving perfectly acceptable disease control (Rusell, 2002).

It should be clarified why the moderate frequency of mutated allele especially in wild types is not reflected *in vitro* sensitivity test. This case can be explained in *V. inaequalis* quantity of the samples that could not be always enough for a precious assesment of the population especially in situation of heterogeneity. Moreover, the material used in *in vitro* and molecular analysis were different and could cause diversity in evaluation. And also,

there could be non viable conidia in *in vitro* test, while qPCR can also detect mutated alleles with non viable fungal material.

The populations collected from orchards where strobilurin used and a poor control noticed, showed lower sensitivity to trifloxystrobin and they were classified as resistant. They presented EC_{50} max from 0.17 to >10 mg L^{-1} and RG from 11.4 to 97.3% and generally high R-alleles frequency was found ranging from 23.01 to 99.54 %. It was observed that in populations defined as resistant *in vitro* test have almost always have high mutated allele frequency. However, in spite of the relatively low frequency of the mutation (5.97% and 0.015%), reduction of the efficacy of trifloxystrobin was observed in only two samples, n.161 and 201 (EC_{50} max 2.56, RG 31.4% and EC_{50} max 0.19 mg L^{-1}, RG 14.6%, respectively). This difference can be indicated the presence of other mechanisms causing reduced sensitivies. Indeed, the mutation does not always explain the QoI-resistant phenotype. It may be due to the alternative respiration pathway. Although it was not observed an influence in this respiration pathway by adding SHAM for testing the sensitivity of some populations and monoconidal isolates in this study, even if a moderate *in vitro* action of SHAM was sometimes noted (Olaya *et al.*, 1998) but this activity has been considered of little importance under field conditions (Fernandez-Ortuno *et al*, 2008). Moreover, the other effective mechanisms responsible for QoI resistance remain to be characterized (Steinfield *et al*., 2001; Fernandez-Ortuno *et al*, 2008) and it could be clarified with studies on biochemical and genetic aspects. Many basic aspects on QoI resistance (e.g. genetic stability, segregation) are still not well understood (Gisi and Sierotzki, 2008). Therefore, further experiments to identify additional mechanisms of resistance may also be needed in the future.

Also in all samples collected from orchards with poor scab control probably caused by strobilurins in Turkey in 2011, molecular assays demonstrated the presence of G143A subsititution conferring resistance at a very high level (46.62-99.76%)

Sensitivities of monoconidial isolates of *V. inaequalis*, originated from locations with different history of fungicide usage, were tested to evaluate the sensitivity to QoI. The results showed that the sensitivity of conidial germination of monoconidial isolates from the wild-type and the well controlled populations was also considerably higher than that of the isolates obtained from the populations of the orchards with control failures probably by strobilurins confirmed by high values in percentages of mutyated alleles.

All QoI-sensitive apple scab monoconidial isolates tested was showed very low mutated allele frequency (<1.66 %). In only very few monoconidial isolates obtained from well-controlled populations and wild type population, moderatley high germination at maximum concentration were observed with very low percentage of R-allele. Cellophane method has been used by many researchers for conidial production and to evaluate the spore germination assay of monoconial isolates. However, in this work, EC_{50} values of monoconidial isolates of sensitive populations (especially wild type) were found higher than EC_{50} values obtained by their population in conidial germination test. It is possible that part of these differences is due to cellophane method; indeed in some isolates a part of conidia (from 1 to 10%) were pre-germinated on cellophane plate. However, also in these situations , the sensitivity to trifloxystrobin of monoconidial isolates obtained from sensitive populations were still high (mean EC_{50} value < 0.04 mg L^{-1}). Furtermore, germination (from 1 to 10%) was observed before their insemination on amended petri dishes, also Steinfeld *et al.*, observed the germinated conidia (<10%) when starting the experiments (2001). So their EC_{50} values after insemination were clearly higher respect to the ones of correspondent populations. The conidial suspensions of some tested isolates were inoculated on apple seedlings to test the sensitivity of germinating conidia in order to conserve the sporulation in dried ambient and avoid the anticipated germination. In tested these isolates, sensitivity to trifloxystrobin was found higher in *in vitro* tests with conidia obtained from inoculated

leaves. However, all isolates were not inoculated on seedlings because of low germination capacity and conidia production of monoconidial isolates on seedlings and besides that such a procedure is extremely time and labor consuming, especially when a high number of isolates need to be assessed and the tests need to be repeated. Moreover, fungicide testing also requires a sufficient amount of sporulating fungal material. In addition, it was detected high RG 23.3% and 45.47% (EC_{50} 0.03 and 0.38 mg L^{-1}, respectively) in only two monoconidal isolates from well controlled population and 24.79% RG (EC_{50} 0.004 mg L^{-1}) in one wild type monoconidial isolate with almost 0% mutated allele (<0.1%). This high relative germination value can be also explained by other resistance mechanisms which mentioned above.

The results obtained *in vitro* demonstrated that trifloxystrobin, showed very low sensitivity against all resistant isolates used in the study. Fungicide sensitivity testing linked with cytochrome b sequence analysis showed that high levels of QoI resistance in resistant monoocnidal isolates of *V. inaequalis* always associated with the presence of a specific mutation in the mitochondrial cytochrome b gene with high mutated allele frequency (>98.43). The activity of trifloxystrobin against mutant isolates was noticeably lower compared to wild-type isolates. It was concluded that there is strong evidence that the point mutation found in single resistant isolates of *V.inaequalis* is a major cause of resistance to trifloxystrobin.

Results from these studies showed that trifloxystrobin is sometimes more effective against conidia than mycelial growth *in vitro*, in some cases the activity is similar. It is recommended that sensitivity studies be conducted on conidia rather than mycelia, because the activity of strobilurins is mainly based on germination (Olaya and Koller, 1999b; Barlet *et al.*, 2002).

It was noted that there were no significant variations in allele frequencies within a tested single spore iolates of populations.

It was decided to use the same material in biological assays and in molecular assays to evaluate the sensitivity and detect the mutated allele of monoconidial isolates. Therefore, we have established an efficient method utilizing Chelex 100 for extraction DNA from *Venturia inaequalis* spores of monoconidial isolates tested in biological assay. Sometimes, it was difficult to get amplifications from PCR at the first time with a Chelex extract, second extraction of extracted product was needed to be done twice. We were able to obtain good yield of DNA from *Venturia inaequalis* spores by Chelex 100. Thus, this extraction method developed in this study is feasible and quick to obtain DNA from monoconial isolates and to use in molecular analysis. To analyse the presence and detection of point mutation of *V. inaequalis* monoconidial isolates, mycelial extraction has been used by many researchers using CTAB based method. However, this procedure is very time consuming comparing Chelex 100 extarction done in 2-3 hours.

A comparison was made between conventional and qPCR assessment of fungicide activities. The study confirmed the applicability of qPCR assay to efficiently determine the strobilurin-resistance level in apple orchards by comparing it with the conventional method.

In this study, quantitative assessments using qPCR followed a similar pattern to that obtained using *in vitro* conidial germination test in very sensitive and very resistant populations. In fact, in most cases, it was noticed when RG was < 10%, mutated allele frequency was < 10% and when RG was > 70%, very high mutated allele (>80%) was detected. However, some variability between two test results was observed in hetereogenous populations. Therefore, the results of correlations between *in vitro* and qPCR showed a positive but not very high correlation for *Venturia inaequalis* populations (R^2=0.70). On the contrary, this correlation between two assessment methods was very high for monoconidial isolates (R^2=0.92). qPCR assessment was highly representative of the results obtained by *in vitro* assay for monoconial isolates. Because almost all monoconidal isolates showed a very high sensitivity or very low sensitivity to trifloxystrobin in both assays. Detecting high

correlation value between both assessments confirmed the accuracy of qPCR assessment for very resistant populations and also often for very sensitive populations.

The method developed here was designed as alternative to tradional methods and qualitative tests and showed a better sensitivity than the CAPS method and *in vitro* tests. However, not a very high correlation between biological and molecular data was observed in intermediate frequency values, as represented by hetereogenous populations. Moreover, examing the only a rather small part of the whole fungal material which present in an orchard or on apple tree, in some cases, can cause different results on populations between the two methodologies and an imprecise evaluation. Michalecka *et al*., 2011, advised to increase the number of repetitions of every samples in qPCR to obtain a reliable assesment of the allele ratio in field populations. Also *in vitro* tests with more multiplicated conidia could be useful for examination. Moreover, it should be remembered that qPCR can also detect the mutation from non-viable conidia with viable ones while *in vitro* tests is relied only on viable conidia.

Also these differences in examined material by the two methodologies, in some cases, may cause differences in results on populations, while the problem seems to have a much lower importance on monoconidial isolates. The hetereogenicity of population can be the main factor that can cause differences between methodologies.

Therefore, in some cases it is difficult to assess the resistance in the field by only qPCR. The results of this study suggest that *in vitro* tests combined with qPCR assay are suitable for assessing the risk of *V. inaequalis* at the field level.

6. Conclusion

A method to determine the development of fungicide resistance in apple scab populations is a basic step in being able to predict possible trends in fungicide resistance on a large scale. The qPCR assay described in this study is applicable for high-throughput detection of the resistant A143 allele in more than 60 populations. Often it is necessary to detect the presence of a pathogen early and quickly, and to determine the frequencies of resistant and sensitive fungal pathogen isolates in one ore more regions. The most commonly used test for determining QoI sensitivity of *V. inaequalis* was a biological tests conducted at several concentrations and was very fastidious. Also, conventional methods to detect resistance are time-consuming and labor-intensive. Once, the resistance mechanism is known, it is often faster to use molecular detection. The main advantage of molecular real-time methods over pre-existing, is their high sensitivity and qPCR allows for the detection of small amounts of the desired allele in bulk samples, thus eliminating the need to maintain pure cultures of the pathogens. This is definitely less time-consuming than *in vitro* sensitivity growth testing and more useful, especially when traditional tests are impossible to conduct.

This study confirmed the applicability of qPCR assay to efficiently determine the strobilurin-resistance level in apple orchards by comparing it with the conventional method. The results generally demonstrated a good correlation between the allele-specific gene frequencies determined by qPCR and the well-established quantitative biological tests such as conidial germination. However, it was observed that using qPCR method makes it possible to measure the mutation level in DNA isolated from viable and non-viable fungal material. Therefore, results obtained in quantitative PCR and from traditional spore germination assay can differ for the same fungal population. Moreover, the mutation does

not always explain the QoI-resistant phenotype. They may be due to other mechanisms causing reduced sensitivies which is necessearry to be characterized.

qPCR allows a rapid detection of mutation with known resistance mechanisms, at low frequencies, and even if it is not possible to have a precise correlation between biological and molecular data, it is possible to observe that populations classified as sensitive *in vitro* tests have generally a rather precise range of percentage of mutated alleles, and the same can be observed on resistant populations where the percentage of mutated alleles is generally superior to certain values. However, it is not always possible to correlate the frequency of detection of the mutation with biological assessment.

In conclusion, in such situations monitoring by molecular techniques must be supported by standard *in-vitro* resistance assessments and observation of field performance in order to have a more reliable conclusion on sensitivity of each *V. inaequalis* population to strobilurins.

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