Giuliana Maddalena
Plant Disease

Absence of Fitness Trade-Offs in Fungicide-Resistant *Venturia inaequalis*: Evidence Along an Altitudinal Gradient

G. Maddalena[1*], B. Lecchi[1], M. Peracchi[1], G. Pigni[1], G. Russo[2], and S.L. Toffolatti[1]

[1]Department of Agricultural and Environmental Sciences, University of Milan, Milan, Italy, 20133.

[2]Ordine dei Dottori Agronomi e Forestali di Milano, Milano, Italy, 20131.

Corresponding author: G. Maddalena; Email: giuliana.maddalena@unimi.it

Keywords: apple scab, fungicide resistance, fitness penalties, environmental influence

Abstract

G. Maddalena[1*], B. Lecchi[1], M. Peracchi[1], G. Pigni[1], G. Russo[2], and S.L. Toffolatti[1]. Absence of Fitness Trade-Offs in Fungicide-Resistant *Venturia inaequalis*: Evidence Along an Altitudinal Gradient. Plant Dis. (XX:XX-XX).

Venturia inaequalis, a hemibiotrophic ascomycete, is the causal agent of apple scab, a major disease affecting apple production worldwide. The widespread use of fungicides in orchard management has led to the selection of resistant strains. To limit the spread of these resistant strains, it is essential to understand their competitive fitness within the population. In this study, we evaluated the resistance profiles and fitness components of 23 *V. inaequalis* isolates from both treated and untreated orchards in Northern Italy, focusing on five fungicides: dodine, cyprodinil, trifloxystrobin, boscalid, and myclobutanil. Fitness parameters, including mycelial growth, conidia number, and conidia size, were assessed in relation to fungicide resistance and environmental factors, such as altitude. The results revealed that, overall, resistant and sensitive strains showed no significant differences in fitness, except for cyprodinil-resistant strains, which exhibited enhanced mycelial growth and increased conidia size, and dodine-resistant strains, which produced smaller conidia. Additionally, altitude influenced conidial size, with higher elevation sites correlating with smaller conidia. These findings suggest that both genetic and environmental factors contribute to conidial variation, which may impact pathogen dispersal and infection dynamics. This study highlights the resilience and potential for spread of fungicide-resistant strains and underscores the need for integrated resistance management strategies to maintain sustainable and high quality apple production.

Keywords: apple scab, fungicide resistance, fitness penalties, environmental influence

Apple scab, caused by the hemibiotrophic fungus *Venturia inaequalis*, is the most economically significant disease affecting apple worldwide (Belete and Boyraz 2017). The pathogen is widespread in commercial apple-growing regions and causes characteristic lesions on leaves and fruit, leading to yield losses of up to 70% (Bowen et al. 2011). *V. inaequalis* overwinters in fallen leaves, where sexual ascospores develop and initiate primary infections in spring (MacHardy 1994). Asexual conidia facilitate secondary infections throughout the growing season, particularly in temperate regions with cool, moist springs. Italy is one of Europe leading apple producers, with an estimated 2.3 million tonnes produced in 2022 (Eurostat). The economic impact of apple scab is severe, as even low infection levels render fruit unmarketable (MacHardy 1996), necessitating intensive fungicide applications (15–25 per season) to manage the disease (Holb et al. 2017). However, *V. inaequalis* poses a high risk for fungicide resistance development (Fungicide Resistance Action Committee (FRAC) 2019), with resistant isolates documented in multiple regions, including Italy (Toffolatti et al. 2016). Cases of resistance to dodine, demethylation inhibitor (DMI), quinone outside inhibitor (QoI), anilinopyrimidine (AP), and succinate dehydrogenase inhibitor (SDHI) fungicides have been reported (Beresford et al. 2012; Fiaccadori et al. 2011; Köller et al. 2004). The emergence of multi-resistant strains in the USA, Greece, and Turkey further complicates management (Chapman et al. 2011; Chatzidimopoulos et al. 2022).

Fungicide resistance evolution is influenced by factors such as selection pressure, host susceptibility, application regimes, and fungicide persistence (Peever and Milgroom 1994). A key determinant of resistance stability is the fitness of resistant isolates, which may exhibit trade-offs in growth and sporulation compared to sensitive strains (Mayr 2001). Assessing

fitness components can improve predictions of pathogen population dynamics and the durability of resistance (Avenot and Michailides 2010). This study characterized 23 *V. inaequalis* strains from Northern Italy (Lombardy region), collected from treated and untreated orchards, to evaluate: i) resistance to five fungicides (dodine, cyprodinil, trifloxystrobin, boscalid, myclobutanil) of different resistance classes; and ii) fitness components (mycelial growth, conidia production, and size) in relation to resistance profiles and environmental conditions of sampling site (altitude). The findings provide insights into the spread and persistence of resistant *V. inaequalis* populations.

Materials and Methods

Collection of field isolates. The sampling strategy was designed to ensure comprehensive coverage of the *V. inaequalis* population across both fungicide-treated and untreated orchards, while considering environmental variability. A total of 30 orchards were included in the study, with 20 regularly treated with fungicides and 10 that had never been treated. Over three consecutive years, 50 infected leaves showing visible apple scab lesions were collected in October from each of the 20 treated orchards located in Lombardy, Northern Italy. Additionally, samples were gathered from the 10 untreated orchards. 50 leaves were randomly selected from different sections of the trees within each orchard. This method ensured a representative distribution of leaves affected by apple scab disease, accounting for natural variability, including differing exposure to fungicide treatments. All samples were placed in paper bags and stored at 4°C until further analysis. This approach provided a robust dataset for evaluating the presence of fungicide-resistant strains in both treated and untreated

orchards. A sporulating lesion per leaf was removed with a razor blade and scraped on 1.5 % water agar (Bacto Agar, Difco), in order to release conidia. Plates were stored at 20°C overnight to promote the germination of conidia. Individual germinated spores were excised under an optical microscope and transferred to fresh half strength potato dextrose agar (PDA; Difco, 19.5 g/L) medium amended with tetracycline (0.005 %), chloramphenicol (0.01 %) and dichloran (0.002 %). A culture collection consisting of three strains derived from single conidia was established per orchard and maintained on PDA medium at 20°C. For the present study, 23 isolates were randomly selected from this collection (Table 1).

***In vitro* fungicide sensitivity assays.** The isolates were tested for sensitivity to the following fungicides: boscalid (SDHI, 99.5% purity), cyprodinil (AP, 99.19% purity), dodine (96.6% purity), myclobutanil (IBS, 99.67% purity), and trifloxystrobin (QoI, 99.54% purity). All active ingredients (technical grade) were purchased from Sigma-Aldrich (Milano, Italy). Myclobutanil was selected for this study because it was one of the most widely used DMI fungicides in apple scab management for many years, and its inclusion allows to assess the impact of past fungicide use on the sensitivity of *V. inaequalis* populations. Although this compound is no longer applied in the field and its activity is strongly affected by DMI-shifting, it was included as a representative molecule of the class. Since the isolates tested are no longer viable, assays with newer DMIs (e.g. difenoconazole or mefentrifluconazole) could not be performed. Therefore, the results obtained with myclobutanil should not be interpreted as indicative of the current field efficacy of DMIs, but rather as providing insights into historical selection pressure and cross-resistance patterns within this fungicide class.

Trifloxystrobin, myclobutanil, and cyprodinil were dissolved in dimethyl sulfoxide (DMSO),

dodine in n-butanol, and boscalid in acetone to a concentration of 10 g/L, then diluted to 1 g/L with sterile distilled water. The concentrations of organic solvents used for fungicide dissolution had no effect on the growth of *V. inaequalis* (data not shown). Fungicides were added to the media before pouring (PDA for all fungicides, except cyprodinil, which was added to a minimal medium lacking methionine, following De Miccolis Angelini et al. 2014). Trifloxystrobin medium was amended with 100 mg/L salicylhydroxamic acid (SHAM) (Olaya et al. 1998)to inhibit the cyanide-insensitive respiration pathway (alternative respiration). Control media without fungicides were also prepared for each assay. The concentrations of fungicides used are listed in Table 2.

For each *V. inaequalis* isolate, three Petri dishes were prepared for each fungicide concentration. Each dish was inoculated with three mycelial plugs (5 mm in diameter), taken from the actively growing edge of the colony and placed equidistantly on the agar surface. After four weeks of incubation at 20°C in the dark, radial growth was measured as the mean of two perpendicular colony diameters. Growth inhibition (GI) at each fungicide concentration was calculated as the percentage reduction in radial growth on fungicide-amended medium relative to the control (non-amended) medium.

Assessment of fitness parameters. Mycelial growth, conidia concentration and size were assessed *in vitro* as indicators of strain fitness (Chapman et al. 2011). For each isolate, three mycelial plugs (5 mm in diameter) were excised from the margin of actively growing colonies on a previous PDA plate and placed on a single PDA plate covered with a cellophane membrane. Each isolate was inoculated onto three replicate plates, resulting in a total of nine

plugs per isolate. After incubation at 20 °C in the dark for four weeks, radial growth was measured as previously described.

Following diameter measurements, the mycelium was scraped from the cellophane, transferred into a 15 mL sterile tube containing 5 mL of sterile distilled water, and shaken to detach the conidia. The suspension was filtered through three layers of sterile gauze to remove mycelial debris and centrifuged at 11,000 rpm for 6 minutes. The supernatant was discarded, and the conidia were resuspended in 1 ml sterile distilled water and vortexed for 3 minutes to disperse conidial chains and obtain a homogenous suspension. Conidial concentration(conidia/mL) was determined using a KOVA counting chamber under a Zeiss Primo Vert optical microscope (Zeiss).

A colony was defined as the mycelial growth originating from a single mycelial plug on a PDA plate. Conidial size, expressed as area (mm^2), was determined by image analysis. For each colony, six conidia were randomly selected and measured, providing a representative assessment of size variability while maintaining methodological efficiency. Since three colonies were analyzed per isolate a total of 18 conidia were measured per isolate. Images were processed using GIMP 2.10 (GNU Image Manipulation Program) by converting the background to white and the conidia to black. The black area was quantified in pixels and converted to mm^2 using the calibration factor (pixels per mm) according to the following formula:

$$Conidial\ area\ (mm^2) = \left(\frac{Area\ in\ pixels}{Pixels\ per\ mm}\right)^2$$

where “area in pixels” refers to measured conidial area obtained from the image analysis, and "pixels per millimeter" is the calibration factor determined during the software

setup (0.0020625 pixels per mm). Image analysis allowed for precise and standardized measurements across all colonies and treatments.

Data Analysis. The effective concentration required to inhibit 50% of mycelial growth (EC_{50}) for each isolate was estimated by linear regression of probit-transformed growth inhibition (GI) values against the logarithm of fungicide concentrations (Li et al. 2015), using SPSS v.28 (IBM, Milan, Italy).

Strains were classified as resistant based on EC_{50} thresholds defined for each fungicide: >24 mg/mL for trifloxystrobin (with intermediate-resistant strains ranging from 5 to 24 mg/mL), >7.6 mg/mL for myclobutanil (with intermediate resistance between 5 and 7.6 mg/mL), >3.9 mg/mL for dodine, >12 mg/mL for boscalid, and >0.6 mg/mL for cyprodinil (Toffolatti et al. 2016).

To investigate potential differences in fitness components among isolates with contrasting fungicide sensitivity profiles, a series of statistical analyses was conducted using R v.4.4.2 (R Foundation for Statistical Computing, Vienna, Austria).Residuals from preliminary models were tested for normality using the Shapiro–Wilk test (shapiro.test () function, base R. As the assumption of normality was not met, nonparametric methods were applied. Specifically, differences in mycelial growth, conidial production, and conidial size between resistant and sensitive isolates were assessed using the Mann–Whitney U test (wilcox.test () function, base R). The influence of the altitude of origin (plain, hill, or mountain) on the same fitness components was also evaluated using this nonparametric approach. Statistical significance was determined at $P < 0.05$. Spearman's rank correlation analysis was performed to assess the relationships among mycelial growth, conidial production, and conidial size.

Correlation coefficients (ρ) and their associated p-values were calculated using the rcorr() function from the Hmisc package in R. Results were visualized using the corrplot() function from the corrplot package, where the orientation and color of ellipses indicate the direction of the correlations, while the intensity is represented by their narrowness and shading darkness. Only statistically significant correlations ($p < 0.05$) were displayed in the plot.

Results

Fungicide sensitivity. Table 3 shows the EC_{50} values calculated for each fungicide and the corresponding sensitivity profiles, based on the thresholds reported in the literature.

As shown in Figure 1, the frequency of sensitivity phenotypes among *V. inaequalis* isolates varied depending on the fungicide tested. All isolates were sensitive to boscalid. For dodine, 87% of isolates were classified as sensitive and 13% as resistant. An analogous situation was found for cyprodinil (88% of sensitive and 12% of resistant isolates). In the case of myclobutanil, 82.6% of isolates were sensitive, 8.7% resistant, and 8.7% intermediate-resistant. The highest frequency of resistance was observed for trifloxystrobin, with 41.7% of isolates classified as sensitive, 45.8% as resistant, and 12.5% as intermediate.

No isolates exhibited resistance to all five fungicides tested. However, only 8% of strains were sensitive to all five fungicides, indicating that 92% were characterized by resistance to at least one fungicide. Three isolates showed multiple resistance, being resistant to two fungicides simultaneously. Specifically, isolate 319 was resistant to dodine and trifloxystrobin, isolate 366 to myclobutanil and trifloxystrobin, and isolate 370 to cyprodinil and trifloxystrobin (Table 4). Notably, trifloxystrobin resistance was common to all three isolates.

Fitness components. No significant differences in mycelial growth were observed between isolates grouped by resistance to a single chemical class and sensitive isolates ($P > 0.107$), except for cyprodinil. Resistant isolates to cyprodinil exhibited significantly greater mycelial expansion than sensitive ones ($P = 0.003$) (Figure 2).

No significant differences ($P > 0.073$) were found between sensitive and resistant isolates for conidial concentration, except for trifloxystrobin, where intermediate-resistant isolates had higher conidial production ($P = 0.022$) than sensitive isolates. Resistant isolates exhibited intermediate behavior in terms of conidial production.

Significant differences in conidial size were observed between sensitive and resistant isolates for dodine and cyprodinil. Dodine-resistant isolates produced smaller conidia than sensitive ones ($P = 0.002$), while cyprodinil-resistant isolates produced larger conidia than sensitive ones ($P = 0.043$).

To assess whether multiple resistance is associated with differences in fitness-related traits, the average values of mycelial diameter (mm), conidial concentration (conidia/mL), and conidial size (mm^2) were compared between three fully sensitive strains (S6, 362, and 381) and three multiple-resistant strains (319, 366, and 370). No significant differences were observed for any of these parameters (Figure 3).

After assessing whether fitness-related traits were influenced by the resistance profiles, the potential impact of an environmental factor on the same traits was further investigated. In particular, attention was focused on the altitude of the sampling site, selected as a proxy for environmental variability. The effect of sampling site altitude on mycelial growth, conidial production, and conidial size was therefore evaluated. Based on the altitude of the sampling

sites (Table 1), the isolates were divided into two categories: hill (<600 m a.s.l.) and mountain (>600 m a.s.l.). Radial growth of *V. inaequalis* isolates after 4 weeks of incubation on PDA medium ranged from 20.77 mm to 38.67 mm, with no significant differences observed between strains isolated from different altitudes ($P = 0.86$) (Figure 4-A). Considerable variations in conidial concentration and size were observed among isolates from different altitudes (Figure 4-B and C). Sporulation rate significantly increased with altitude, ranging from 0.43×10^3 to 132.2×10^3 conidia/mL. Isolates sampled from mountain sites produced the highest conidial concentrations, whereas isolates from hill sites produced significantly fewer conidia ($P = 0.022$) (Figure 4-B). Conversely, conidial size significantly decreased with increasing altitude: isolates from mountain sites produced smaller conidia compared to those from lower altitudes ($P = 0.012$).

The correlation matrix presented in Figure 5 illustrates the relationships among key traits associated with pathogen fitness: mycelial colony diameter, conidial concentration, and conidial size.

No significant correlation was found between mycelial diameter and conidial size ($\rho = 0.343$, $P > 0.10$), nor between mycelial diameter and conidial number ($\rho = -0.313$, $P > 0.10$). In contrast, a significant negative correlation was found between conidia number and size ($\rho = -0.433$, $P = 0.039$).

Discussion

The emergence and persistence of fungicide-resistant *V. inaequalis* strains represent a significant threat to apple production. This study assessed the fungicide sensitivity and fitness

components of 23 isolates collected from Northern Italian orchards, revealing a variable resistance landscape and important insights into the ecological behavior of resistant strains.

The results demonstrate that resistance to one or more fungicides does not necessarily entail a fitness cost. In the majority of cases, no significant fitness costs were detected, as no notable differences in mycelial growth, conidial production, or conidium size were observed between different sensitivity profiles—except for specific traits linked to certain fungicide resistance types. These findings suggest that resistance traits may persist in field populations even in the absence of fungicide-driven selection pressure. This aligns with previous observations that genetic background often plays a more decisive role in fungal fitness than the resistance phenotype itself (Avenot and Michailides 2010; Martinez et al. 2005).

Resistance to trifloxystrobin was the most prevalent among the fungicides tested, with 58.3% of isolates displaying intermediate or full resistance. While no significant fitness advantage or disadvantage was associated with full resistance, isolates classified as intermediate-resistant showed increased conidial production. This suggests that partial resistance may confer subtle biological advantages under certain conditions, warranting further investigation into the ecological behavior of such phenotypes (Mikaberidze et al. 2025). This aligns with previous studies showing that QoI resistance in *V. inaequalis*, while leading to loss of fungicide efficacy, has generally not been associated with measurable fitness costs (Weber et al. 2025).

Resistance to cyprodinil, an anilinopyrimidine fungicide, was detected in about 12% of isolates. Notably, cyprodinil-resistant strains exhibited significantly greater mycelial growth and conidial size than sensitive ones, indicating a possible selective advantage. These findings are

consistent with reports in other fungal species, such as *Alternaria alternata* and *Botrytis cinerea*, where resistance to AP fungicides did not incur observable fitness penalties (Avenot and Michailides 2015).

In contrast, dodine-resistant isolates produced significantly smaller conidia than sensitive strains, although no differences were observed in mycelial growth or conidial output. This observation may reflect a pleiotropic effect of dodine resistance. Dodine is a guanidine fungicide that interferes with membrane integrity and may disrupt endocytic or signaling pathways involved in fungal morphogenesis (Schuster and Steinberg 2020). Adaptations conferring resistance to such compounds could alter the regulation of cell wall synthesis, cytoskeletal dynamics, or energy allocation, leading to the production of morphologically altered conidia. Although smaller conidia may not directly impact total spore output, they could influence key epidemiological traits such as dispersal capacity, environmental resilience, or infectivity. Modeling studies suggest that smaller fungal spores remain airborne longer and disperse farther, while larger spores tend to settle more quickly, highlighting a trade-off between dispersal potential and deposition efficiency (Golan and Pringle 2017). Empirical evidence from other fungal pathogens supports this pattern, showing that spore size can modulate dispersal and infection probability depending on the environmental context (Norros et al. 2014). Additionally, in some fungal pathogens, such as *Histoplasma capsulatum*, smaller microconidia have greater infectivity potential due to their ability to access host tissues more effectively (Wang and Lin 2012). Indeed, spore size is known to affect survival during aerial dispersal: larger spores generally show greater tolerance to environmental stresses such as desiccation and UV radiation, while smaller spores might be more susceptible but could remain

airborne longer due to their lower mass (Golan et al. 2023). Thus, the reduction in conidial size among dodine-resistant isolates could represent a fitness trade-off, whereby the adaptive benefit of resistance under fungicide pressure is counterbalanced by morphological or physiological constraints.

Boscalid resistance was not observed in any of the isolates tested, despite its widespread use. These findings are in line with previous European monitoring reports, which show that SDHI resistance in *V. inaequalis* is sporadic and has not established in field populations over the last decade (Hoffmeister et al. 2024; Weber et al. 2025). Our data confirm the apparent stability of SDHI sensitivity in Northern Italy, further supporting the hypothesis that current SDHI resistance mechanisms in *V. inaequalis* may impose significant fitness costs, limiting their persistence in natural populations. Resistance to myclobutanil was detected at low levels (17.4%) and showed no apparent impact on fitness traits. The choice of myclobutanil as test compound was motivated by its historical relevance, since it was one of the most extensively used DMIs in apple scab control for decades. For this reason, its inclusion provides insights into how past fungicide applications shaped the sensitivity profile of V. inaequalis populations. It is recognized that myclobutanil is no longer representative of current field efficacy within the DMI class, as resistance to this compound is widespread and sensitivity shifts are well documented. However, its use in this study is informative with respect to cross-resistance dynamics, highlighting the long-term evolutionary response of the pathogen to intensive DMI use. More recently introduced molecules such as difenoconazole or mefentrifluconazole generally retain better activity against *V. inaequalis*, but could not be included here because the tested isolates were no longer viable. Similarly, recent studies on

DMI fungicides have also reported no measurable fitness costs in resistant *V. inaequalis* isolates (Mubashir et al. 2025). These findings emphasize that the ecological success and persistence of fungicide-resistant *V. inaequalis* strains are not solely determined by fitness penalties, but rather by a complex interplay between resistance mechanisms, trait-specific pleiotropic effects, and genetic background. Resistance phenotypes that do not incur measurable fitness costs, or that confer selective advantages in particular traits, may become entrenched in field populations even in the absence of fungicide pressure. This persistence is consistent with recent studies demonstrating that DMI resistance in *V. inaequalis* remains phenotypically stable across multiple transfers without fungicide exposure (Hoffmeister et al. 2024). Similar observations have been reported in *V. inaequalis* populations from Northern Germany, where strains resistant to methyl benzimidazole carbamates (MBC) and QoI remained highly prevalent even in abandoned or unmanaged orchards, highlighting the long-term persistence of resistance without ongoing fungicide applications (Weber et al. 2025). Consequently, resistance management strategies must account for the ecological competitiveness of resistant strains, not just their initial emergence. Beyond fungicide resistance, our data indicate that environmental factors, particularly altitude, strongly influence *V. inaequalis* fitness traits. Although additional ecological parameters may contribute to fitness variation, altitude was chosen as it was consistently available for all isolates and reflects a gradient of climatic conditions (Nakato et al. 2023). Isolates from higher elevations produced significantly more conidia but of smaller size, suggesting a trade-off strategy favoring quantity over size in resource-limited or cooler environments. This is supported by the significant negative correlation between conidial concentration and size. This inverse relationship between spore

size and number has also been observed in other plant pathogens, such as *Plasmopara viticola* and *Phytophthora infestans*, where a higher number of asexual spores is typically associated with smaller individual spores (Mariette et al. 2018). Similar trends have been reported in other plant-pathogenic fungi, where spore morphology and production strategies are modulated by environmental conditions (Norros et al. 2014b; Rangel et al. 2005). This trade-off may represent an adaptive strategy whereby fungal strains optimize dispersal under specific environmental constraints. Smaller conidia produced in greater numbers could enhance aerial dissemination, while larger spores, more common at lower altitudes, might favor adhesion, deposition, or early infection success. Similar patterns have been observed in *V. inaequalis* populations in different habitats, where environmental structure influences spore size and production. In particular, larger spores are produced in lower numbers but may have a higher probability of successful deposition and early infection, whereas smaller spores are more numerous and better dispersed but each with a lower chance of infecting a host (De Gracia et al. 2015). These findings suggest that pathogen fitness may be shaped by distinct growth strategies, where conidial production responds to different selective pressures. Understanding these correlations is crucial for elucidating the pathogen adaptability to diverse environmental conditions and hosts, with implications for disease management and control strategies. These findings emphasize the complex interplay between genetic resistance and ecological adaptation in shaping pathogen fitness.

Overall, the absence of measurable fitness costs in most resistant isolates, including those with multiple resistances, raises concerns about their potential to persist and spread in orchard ecosystems. The fact that fitness components were more strongly influenced by

altitude than by resistance status further highlights the adaptive capacity of *V. inaequalis* populations. This underscores the urgency of integrating anti-resistance measures, such as fungicide rotation, limited applications, and environmental monitoring, into apple scab management programs.

In conclusion, this study provides evidence that fungicide-resistant *V. inaequalis* strains in Northern Italy are capable of maintaining competitive fitness under a variety of environmental conditions. The resilience of these strains, especially in the absence of clear fitness penalties, poses a serious challenge for long-term disease control. Integrated disease management strategies that address both resistance evolution and environmental adaptability will be essential to safeguard fungicide efficacy and sustain apple production.

Literature Cited

Avenot, H. F., and Michailides, T. J. 2010. Progress in understanding molecular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi. Crop Protection 29:643–651. https://doi.org/10.1016/j.cropro.2010.02.019.

Avenot, H. F., and Michailides, T. J. 2015. Detection of isolates of *Alternaria alternata* with multiple-resistance to fludioxonil, cyprodinil, boscalid and pyraclostrobin in California pistachio orchards. Crop Protection 78:214–221. https://doi.org/10.1016/J.CROPRO.2015.09.012.

Belete, T., and Boyraz, N. 2017. Critical review on apple scab (*Venturia inaequalis*) biology, epidemiology, economic importance, management and defense mechanisms to the causal agent. Journal of Plant Physiology & Pathology 5. https://doi.org/10.4172/2329-955x.1000166.

Beresford, R. M., Wright, P. J., Wood, P. N., and Park, N. M. 2012. Managing agrichemical application. www.nzpps.org.

Bowen, J. K., Mesarich, C. H., Bus, V. G. M., Beresford, R. M., Plummer, K. M., and Templeton, M. D. 2011. *Venturia inaequalis*: The causal agent of apple scab. Molecular Plant Pathology 12:105–122. https://doi.org/10.1111/j.1364-3703.2010.00656.x.

Chapman, K. S., Sundin, G. W., and Beckerman, J. L. 2011. Identification of resistance to multiple fungicides in field populations of *Venturia inaequalis*. Plant Disease 95:921–926. https://doi.org/10.1094/PDIS-12-10-0899.

Chatzidimopoulos, M., Zambounis, A., Lioliopoulou, F., and Vellios, E. 2022. Detection of *Venturia inaequalis* isolates with multiple resistance in Greece. Microorganisms 10:2354. https://doi.org/10.3390/microorganisms10122354.

De Gracia, M., Cascales, M., Expert, P., Bellanger, M. N., Le Cam, B., and Lemaire, C. 2015. How did host domestication modify life history traits of its pathogens?. PLOS one, 10, 6. e0122909.

Fiaccadori, R., Cicognani, E., Alberoni, G., Collina, M., and Brunelli, A. 2011. Sensitivity to strobilurin fungicides of Italian *Venturia inaequalis* populations with different origin and scab control. Pest Management Science 67:535–540. https://doi.org/10.1002/ps.2090.

Fungicide Resistance Action Committee (FRAC). 2019. FRAC Pathogen Risk List 2019.

Golan, J.J., and Pringle A. 2017. Long-Distance Dispersal of Fungi. Microbiology Spectrum 5:10.1128/microbiolspec.funk-0047-2016. https://doi.org/10.1128/microbiolspec.funk-0047-2016

Golan, J. J., Lagomarsino Oneto, D., Ding, S., Kessenich, R., Sandler, M., Rush, T. A., Levitis, D., Gevens, A., Seminara, A., and Pringle, A. 2023. Differences in spore size and atmospheric survival shape stark contrasts in the dispersal dynamics of two closely related fungal pathogens. Fungal Ecology 66:101298. https://doi.org/10.1016/j.funeco.2023.101298.

Hoffmeister, M., Scheu, P., Glaab, A., Zito, R., and Stammler, G. 2024. Sensitivity evolution in *Venturia inaequalis* towards SDHIs in comparison to other modes of action. European Journal of Plant Pathology 168:763–773. https://doi.org/10.1007/s10658-023-02798-6.

Holb, I. J., Heijne, B., Withagen, J. C. M., and Jeger, M. J. 2017. Effects of integrated control measures on earthworm populations and leaf litter decomposition in Dutch organic apple orchards. Applied Soil Ecology 112:58–69. https://doi.org/10.1016/j.agee.2005.11.021.

Köller, W., Parker, D. M., Turechek, W. W., and Avila-Adame, C. 2004. A two-phase resistance response of *Venturia inaequalis* populations to the QoI fungicides kresoxim-methyl and trifloxystrobin. Plant Disease 87:184–190. https://doi.org/10.1094/PDIS.2004.88.5.537.

Li, J. L., Liu, X. Y., Xie, J. T., Di, Y. L., and Zhu, F. X. 2015. A comparison of different estimation methods for fungicide EC_{50} and EC_{95} values. Journal of Phytopathology 163:239–244. https://doi.org/10.1111/jph.12312.

MacHardy, W. E. 1994. The dispersal of ascospores of *Venturia inaequalis*. Norwegian Journal of Agricultural Sciences 17:137–148.

MacHardy, W. E. 1996. Apple scab: Biology, epidemiology, and management. St. Paul, MN: American Phytopathological Society (APS Press).

Martinez, F., Dubos, B., and Fermaud, M. 2005. The role of saprotrophy and virulence in the population dynamics of *Botrytis cinerea* in vineyards. Phytopathology 95:692–700. https://doi.org/10.1094/PHYTO-95-0692.

Mariette, N., Kröner, A., Mabon, R., Montarry, J., Marquer, B., Corbière, R., Androdias, A., and Andrivon, D. A. 2018. Trade-off between sporangia size and number exists in the potato late blight pathogen *Phytophthora infestans*, and is not altered by biotic and abiotic factors.

Frontiers in Plant Science 19, 9:1841. doi: 10.3389/fpls.2018.01841. PMID: 30619410; PMCID: PMC6305756.

Mayr, E. 2001. Wu's genic view of speciation. Journal of Evolutionary Biology 14:866–867.

Mikaberidze, A., Gokhale, C. S., Bargués-Ribera, M., and Verma, P. 2025. The cost of fungicide resistance evolution in multi-field plant epidemics. PLOS Sustainability and Transformation 4:e0000178. https://doi.org/10.1371/journal.pstr.0000178.

Mubashir, S.S., Bhat, Z. A., Bhat, M. A., Masoodi, K. Z., Shafi F., Mukhtar, M., Nargis, S., Farhana, W., and Gul, A. 2025. Profiling difenoconazole and flusilazole resistance, fitness penalty and phenotypic stability in *Venturia inaequalis*. Scientific Reports 15, 4855. https://doi.org/10.1038/s41598-025-89536-6

Nakato, G. V., Okonya, J. S., Kantungeko, D., Ocimati, W., Mahuku, G., Legg, J. P., and Blomme, G. 2023. Influence of altitude as a proxy for temperature on key *Musa* pests and diseases in watershed areas of Burundi and Rwanda. Heliyon 9:e13854. https://doi.org/10.1016/j.heliyon.2023.e13854.

Norros, V., Rannik, Ü., Hussein, T., Petäjä, T., Vesala, T., and Ovaskainen, O. 2014. Do small spores disperse further than large spores? Ecology 95:1612–1621. https://doi.org/10.1890/13-0877.1.

Olaya, G., Zheng, D., and Koller, W. 1998. Differential responses of germinating *Venturia inaequalis* conidia to kresoxim-methyl. Pesticide Science 54:230–236. https://doi.org/10.1002/(SICI)1096-9063(1998110)54:3<230::AID-PS815>3.0.CO;2-O

Peever, T. L. M., and Milgroom, M. G. 1994. Lack of correlation between fitness and resistance to sterol biosynthesis-inhibiting fungicides in *Pyrenophora teres*. Phytopathology 84:515–519.

Rangel, D. E. N., Braga, G. U. L., Anderson, A. J., and Roberts, D. W. 2005. Influence of growth environment on tolerance to UV-B radiation, germination speed, and morphology of *Metarhizium anisopliae* var. *acridum* conidia. Journal of Invertebrate Pathology 90:55–58. https://doi.org/10.1016/j.jip.2005.05.005.

Schuster, M., and Steinberg, G. 2020. The fungicide dodine primarily inhibits mitochondrial respiration in *Ustilago maydis*, but also affects plasma membrane integrity and endocytosis, which is not found in *Zymoseptoria tritici*. Fungal Genetics and Biology 142:103414. https://doi.org/10.1016/j.fgb.2020.103414.

Toffolatti, S. L., Venturini, G., and Vercesi, A. 2016. Sensibilità di isolati di *Venturia inaequalis* ai principali fungicidi utilizzati su melo in Lombardia. In: Atti Giornate Fitopatologiche, pp. 337–344.

Wang, L., and Lin, X. 2012. Morphogenesis in fungal pathogenicity: shape, size, and surface. PLoS Pathogogens 8(12):e1003027. doi: 10.1371/journal.ppat.1003027.

Weber, R. W. S., Busch, R., and Wesche, J. 2025. Spatial and temporal aspects of fungicide resistance in *Venturia inaequalis* (apple scab) populations in Northern Germany. BioTech, 14(2): 44. https://doi.org/10.3390/biotech14020044

Figures and Tables

Table 1. *V. inaequalis* strain code, orchard number, sampling site, cultivar, treatments, and altitude (meters above sea level).

Table 2. Fungicides and their concentrations used in sensitivity assay

Table 3. EC_{50} values (mg/L) and sensitivity profiles (SP*) for each fungicide tested against *V. inaequalis* isolates

*S=sensitive; R= resistant; IR= intermediate-resistant.

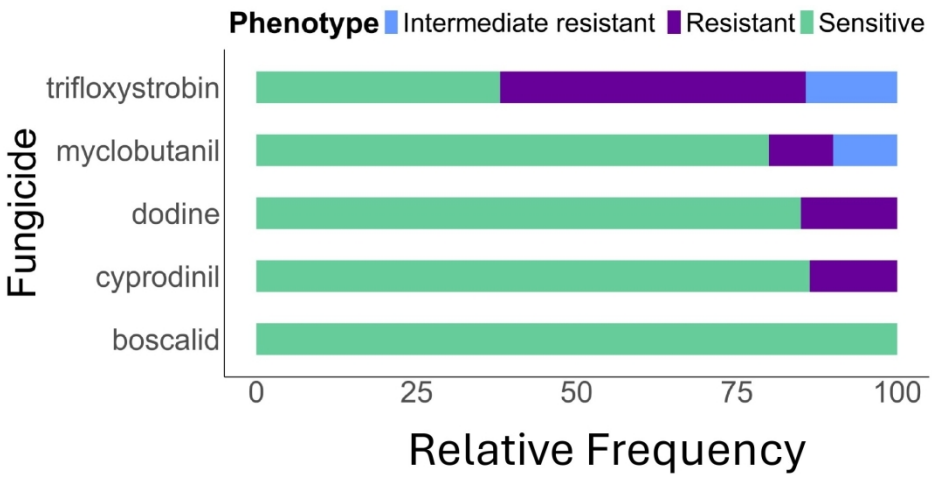
Figure 1. Frequencies of *V. inaequalis* isolates classified as sensitive (green), intermediate resistant (light blue), or resistant (purple) to the five fungicides tested.

Figure 2. Box-plot distribution of (A) mycelial growth, (B) conidial concentration, and (C e D) conidia dimensions for grouped fungicide-resistant and -sensitive *V. inaequalis* isolates showing significant differences. Different letters indicate significant differences among samples ($P < 0.05$).

Figure 3. Box-plot distributions of mycelial diameter (mm), conidial concentration (conidia/mL) and conidial size (mm^2) of three fully sensitive strains (S6, 362, and 381) and three multiple-resistant strains (319, 366 and 370).

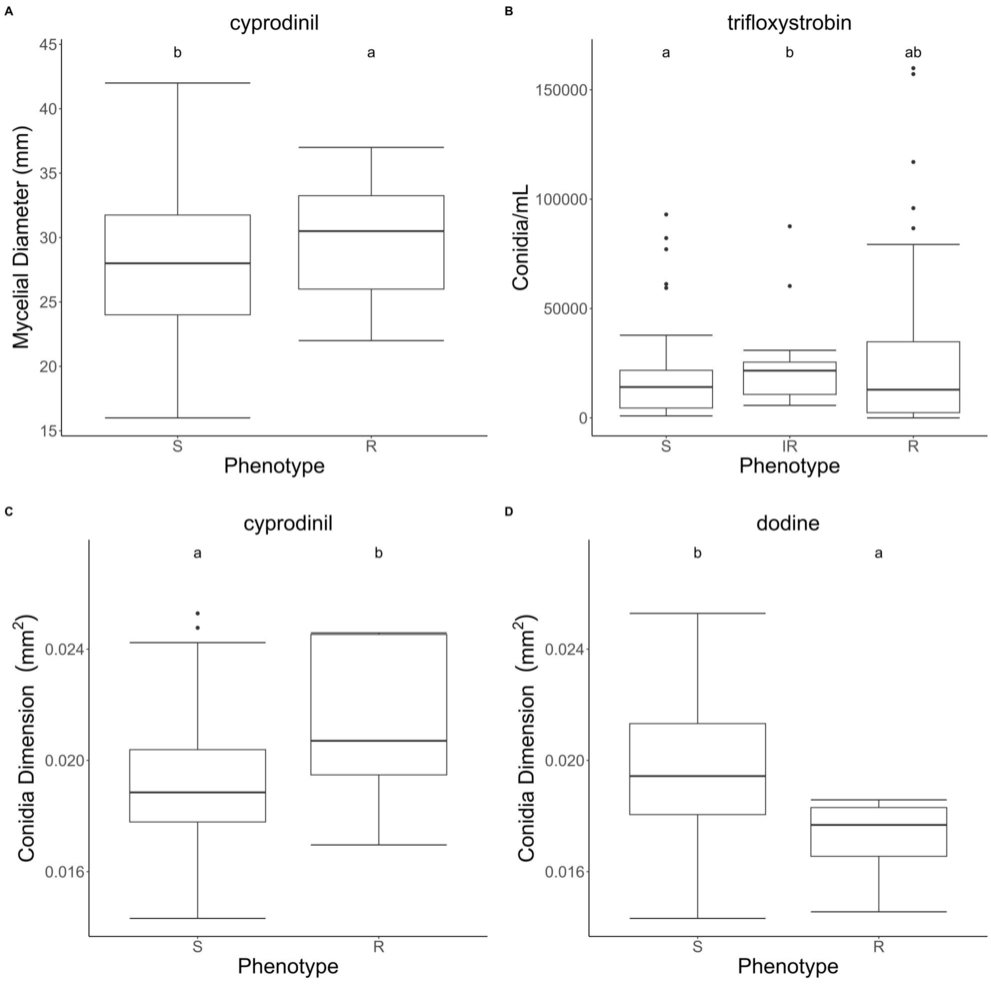
Figure 4. Box-plot distribution of mycelial growth (A), conidial concentration (B), and conidial size (C) for *V. inaequalis* isolates grouped by sampling site altitude. Different letters indicate significant differences among samples ($P < 0.05$).

Figure 5. Correlogram showing Spearman correlation coefficients among the measured variables. The orientation and color of the ellipses indicate the direction of the correlations (blue for positive, red for negative), while the shape and intensity of the ellipses reflect the strength of the correlation. Only statistically significant correlations ($P < 0.05$) are displayed.



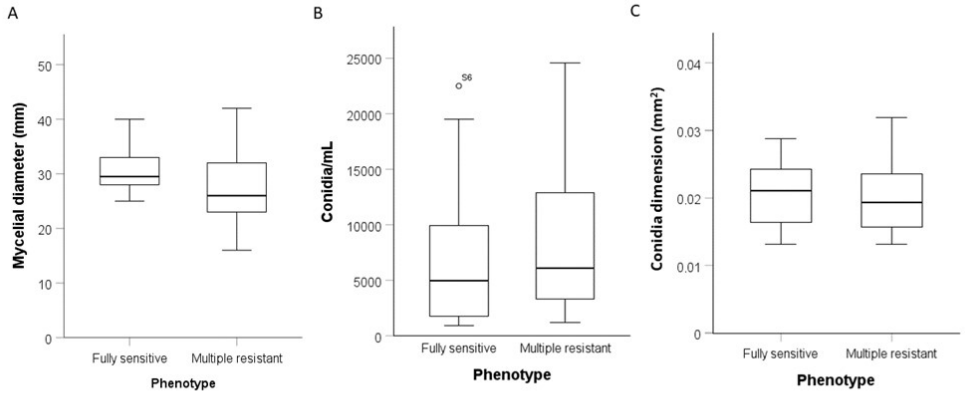
Frequencies of *V. inaequalis* isolates classified as sensitive (green), intermediate resistant (light blue), or resistant (purple) to the five fungicides tested.

183x91mm (300 x 300 DPI)



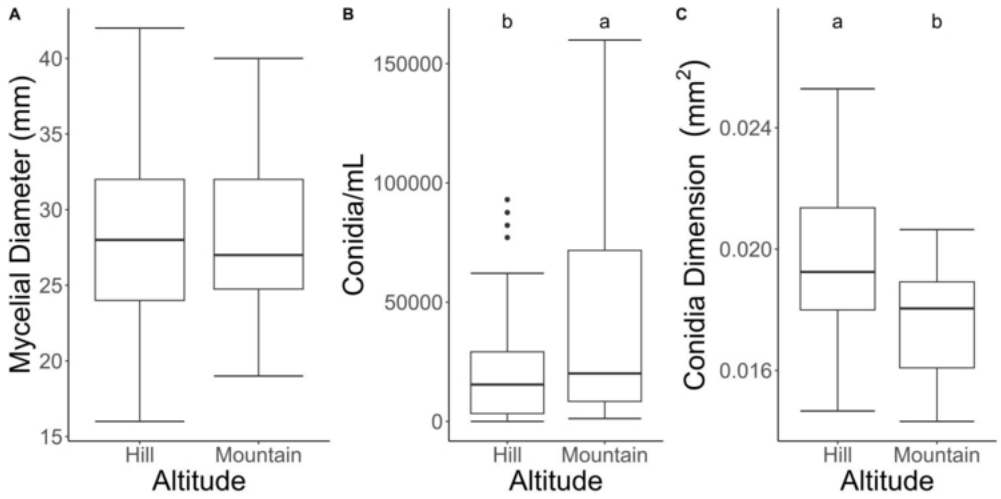
Box-plot distribution of (A) mycelial growth, (B) conidial concentration, and (C e D) conidia dimensions for grouped fungicide-resistant and -sensitive *V. inaequalis* isolates showing significant differences. Different letters indicate significant differences among samples ($P < 0.05$).

42x42mm (600 x 600 DPI)



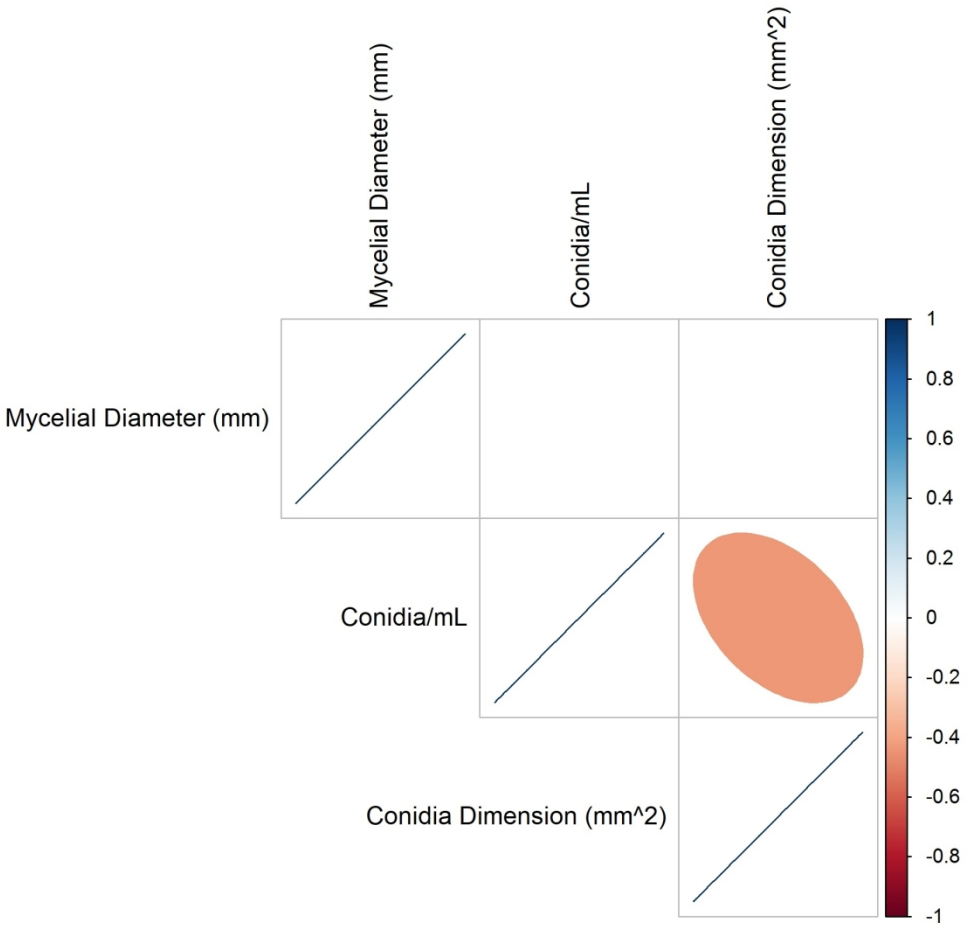
Box-plot distributions of mycelial diameter (mm), conidial concentration (conidia/mL) and conidial size (mm^2) of three fully sensitive strains (S6, 362, and 381) and three multiple-resistant strains (319, 366 and 370).

170x95mm (150 x 150 DPI)



Box-plot distribution of mycelial growth (A), conidial concentration (B), and conidial size (C) for *V. inaequalis* isolates grouped by sampling site altitude. Different letters indicate significant differences among samples ($P < 0.05$).

31x15mm (600 x 600 DPI)



Correlogram showing Spearman correlation coefficients among the measured variables. The orientation and color of the ellipses indicate the direction of the correlations (blue for positive, red for negative), while the shape and intensity of the ellipses reflect the strength of the correlation. Only statistically significant correlations ($P < 0.05$) are displayed.

169x169mm (300 x 300 DPI)

Isolate code	Orchard	Site	Apple cultivar	Treatments	Altitude
51	1	Bagnaria (PV)	Golden	Untreated	Hill (350-600 m a.s.l.)
102, 302	2	Poggiridenti (SO)	Golden + Stark	QoI, dodine, DMI	Mountain (>600 m a.s.l.)
163, 180	3	Tresivio, via Castellanico (SO)	Golden	QoI, dodine, DMI	Hill (350-600 m a.s.l.)
275, 284	4	Tresivio (SO)	Golden+ Red Delicous	DMI	Hill (350-600 m a.s.l.)
299, 301	5	Chiuro, via Tassera (SO)	Red Chief	QoI, dodine, DMI	Hill (350-600 m a.s.l.)
319	6	Surana (SO)	unknown	dodine, AP, DMI	Mountain (>600 m a.s.l.)
327	7	Teglio (SO)	Erovan	Untreated	Mountain (>600 m a.s.l.)
358	8	Ponte in Valtellina (SO)	Granny Smith	Untreated	Hill (350-600 (m a.s.l.)
362	9	Ponte in Valtellina (SO)	Granny Smith	DMI, Pyrimethanil	Hill (350-600 m a.s.l.)
366, 367,368, 370	10	Ponte in Valtellina (SO)	Golden	QoI, dodine, DMI, SDHI, AP	Hill (350-600 m a.s.l.)
371, 372	11	Ponte in Valtellina, via S. Carlo (SO)	Golden	QoI, dodine, DMI, SDHI, AP	Hill (350-600 m a.s.l.)
375	12	Ponte Nizza (PV)	Golden	QoI, dodine, DMI, SDHI, AP	Plain (<350 m a.s.l.)
377	13	Ponte Crenna (PV)	Golden	QoI, DMI	Hill (350-600 m a.s.l.)
381	14	Bagnaria, loc. Zerbo (PV)	Golden	Untreated	Hill (350-600 m a.s.l.)
S6	15	Caslino al Piano (CO)	Golden	Never treated with fungicides	Hill (350-600 m a.s.l.)

Fungicide	Concentrations (mg/L)
Boscalid	0.01, 0.1, 1, 10
Cyprodinil	0.01, 0.1, 1, 10
Dodine	0.005, 0.05, 0.5, 5
Myclobutanil	0.005, 0.05, 0.5, 5
Trifloxistrobin	0.005, 0.05, 0.5, 5

Strair	EC_{50} (mg/l) BOSCALID	SP	EC_{50} (mg/l) CYPRODINIL	SP	EC_{50} (mg/l) DODINE	SP	EC_{50} (mg/l) MYCLOBUTANIL	SP	EC_{50} (mg/l) TRIFLOXYSTROBIN	SP
51	-		-		-		-		0,68	S
102	0.7	S	0.1	S	2.8	S	2.8	S	>100	R
163	-		0.87	R	-		-		-	
180	1	S	0.13	S	2	S	89.6	R	6.7	IR
275	-		0.007	S	-		<0,005	S	>5	IR
284	0.5	S	0.23	S	4.5	R	3.5	S	0.000001	S
299	1.2	S	0.09	S	0.8	S	4.3	S	37.2	R
301	0.2	S	0.07	S	4.8	R	0.2	S	0.0002	S
302	0.1	S	0.23	S	0.7	S	0.4	S	6.3	IR
372	2.6	S	0.32	S	1.9	S	2.4	S	9.8	IR
319	10.9	S	0.32	S	90.66	R	0.4	S	25	R
327	1.3	S	0.03	S	0.7	S	0.011	S	>10	R
358	0.48	S	0.05	S	0.54	S	0.1	S	-	
362	5.1	S	0.06	S	0.9	S	2.9	S	0.0006	S
375	0.5	S	0.09	S	0.3	S	0.27	S	>10	R
366	0.7	S	0.05	S	1.9	S	10	R	>100	R
367	2.2	S	0.07	S	1	S	-	-	0.1	S
368	0.6	S	0.07	S	0.4	S	>5	IR	>100	R
370	0.1	S	>10	R	0.865	S	3	S	>100	R
371	1.1	S	0.22	S	1.3	S	>5	IR	>100	R
377	1.2	S	3.2	R	3.7	S	4.8	S	0.041	S
381	1.5	S	0.08	S	0.5	S	1.5	S	0	S
S6	0.1	S	0.04	S	0.0011	S	<0.005	S	0.0062	S

*S=sensitive; R= resistant; IR= intermediate-resistant.