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Original Article

Apple Scab Disease Severity in the Sais Region of Morocco and its Sensitivity to Three Commercial Fungicides

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Abstract

Apple scab, *Venturia inaequalis* (Cooke) G. Winter, results in numerous fungicide applications in the Sais region of Morocco. We conducted the study to determine the susceptibility of cultivars through field survey and to evaluate the sensitivity of *V. inaequalis* to three fungicides using both *in vitro* and *in vivo* methods. We surveyed 100 apple orchards and collected two samples. Disease survey showed that the cultivars were highly susceptible to apple scab (grand mean of leaf scab: Golden Delicious, 40.31%; Starking Delicious, 20.45%; and Gala, 18.92%). Results underlined no significant differences between isolates in term of inhibition rate of mycelial growth and averaged disease severity. However, both treatment and concentration were statistically significant. EC_{50} values ranged from 2.33 µg/ml to 7.40 µg/ml and resistance factor (RF) values of 0.55, 1.02 and 1.79 were obtained for difenoconazole, trifloxystrobin and thiophanate-methyl, respectively. *In vivo* tests at a concentration of 10 µg/ml on the isolate ViIF using a curative method showed a high efficacy of trifloxystrobin (96.38%), and low efficacy of thiophanate-methyl (29.65%) and difenoconazole (24.62%). *V. inaequalis* was most sensitive to trifloxystrobin followed by difenoconazole and thiophanate-methyl, respectively. From this work, it was recommended testing more isolates and contact fungicides in order to develop a baseline sensitivity of the pathogen population against commonly used fungicides to treat the disease, as continued usage over time and the introduction of new fungal races may result in a decrease in fungicide efficacy.

Keywords: apple cultivars; efficacy; Sais region; sensitivity; susceptibility; *Venturia inaequalis*

Introduction

Apple scab caused by *Venturia inaequalis* (Cooke) G. Winter, is among the most prevalent and economically major diseases in commercial apple orchards in regions with temperate climates worldwide (MacHardy *et al.,* 2001; Belfanti *et al*., 2004; Khajuria *et al.*, 2012; Khajuria *et al*., 2018). The pathogen causes lesions that have a green-olive to velvety-brown coloration when it develops on the leaves and fruits. The lesions become covered with fungal mycelium and spores which is what gives an olive-dark-green colour to the spots. These spots reduce photosynthetic activity and cause young leaf drop (Turechek, 2004). The development of apple scab is influenced by the temperature, the duration of the wetness of leaf, susceptibility of the plant to infection and vegetative growth stage (Belete and Boyraz, 2017). Apple scab may result in losses of up to 70% of the production value if left unchecked, as scabbed fruit are visually unappealing and unfit for sale (Belete and Boyraz, 2017; MacHardy, 2000).

Out of about twenty cultivars of apples grown in Morocco, 'Golden Delicious' occupies up to 50% of the surface area, followed by 'Starking Delicious' (14%) and about 5% of 'Gala' cultivar (MADRPM, 2014). Management of apple scab disease is expensive due to the high number of fungicide applications (12 to 20) (Oukabli, 2004; Moinina *et al*., 2018). In order to prevent the development of fungicide resistance in the pathogen population, a farmer needs to reduce fungicide sprays per season and alternate fungicides with different modes of action (MoA). These are key strategies to prevent the development of fungicide resistance in the pathogen population (Bowen *et al*., 2011). In Morocco, apple farmers

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have over 15 approved active ingredients for apple scab disease control (ONSSA, 2017). The three classes of systemic fungicides most widely used are methyl benzimidazole carbamates (MBC), demethylation inhibitors (DMIs) and quinone outside inhibitors (QoI). The overuse of certain fungicides has led to consumer concerns over their safety, including possible negative health effects. Fungicide application should usually start during the winter season, with copper-based contact fungicides being applied to protect against pathogen infection. In the early spring, when bud swelling and fruit set occur, a second preventive application should be done (Oukabli, 2004). Despite recommendations to apply fungicides early as a preventive measure, the use of fungicides later in the growing season when symptoms have already appeared has increased drastically.

The MoA of each fungicide group may differ from one another depending on its chemical class. MBC fungicides (e.g. thiophanate-methyl) prevent nuclear division by blocking the polymerization of tubulin that affects mitosis (Zhou *et al*., 2016). The fungicidal activity of DMIs is due to an inhibition of fungal synthesis of cytochrome P-450-(CYP) -enzyme ergosterol (Fishel, 2005) with high levels of protective and curative efficacy against *V. inaequalis* (Villani *et al*., 2015). Trifloxystrobin inhibits spore germination and blocks electron transfer at the site of quinol oxidation (the Qo site) in the cytochrome bc1 enzyme complex in the respiratory chain of fungal mitochondria (Köller *et al*., 2004; Bolton *et al*., 2013). A common active ingredient of strobilurin fungicides used in apple production is trifloxystrobin (Chapman *et al*., 2011).

The sensitivity of *V. inaequalis* to fungicides used in controlling apple scab has not been investigated in Morocco. Investigating the sensitivity of pathogens to fungicides is of great importance for crop protection. Furthermore, knowledge of sensitivity parameters will help prevent resistance of *V. inaequalis* to commonly used fungicide groups from occurring. Therefore, the specific objectives of our study were: (i) to determine the difference in the susceptibility of three popular cultivars through field survey, (ii) to evaluate the sensitivity of *V. inaequalis* to difenoconazole, thiophanate-methyl and trifloxystrobin in Ifrane, the apple producing province in the Sais region of Morocco, and (iii) to determine the severity of apple scab on detached leaves before and after fungicide treatments under *in vivo* conditions.

Materials and Methods

Quantification of disease incidence and severity of apple scab in the field

A total of 100 commercial apple orchards were surveyed during the fruit set stage in spring 2018 in the seven districts: 8 from Ain Leuh, 13 from Ait Naamane, 23 from Ait Sbaa, 8 Dayat Aoua, 24 from Laanoucer, 11 from Sidi El Makhfi and 13 from Tigrigra. We assessed the severity of apple scab by comparing sampled leaves to the standard scales. To do this, trees were keenly observed at random from each orchard. Disease incidence and severity were assessed on apple trees and leaves (Fig. 1A) with visible symptoms respectively. Disease incidence, the proportion of infected trees with at least one lesion on a leaf, was calculated:

$$P=n/N \times 100 \quad (1)$$

(P - Disease incidence expressed in percentage, n - number of attacked trees, N - total number of investigated trees). The disease severity was scored as percentage plant leaf area infected with scab according to a scale of 0 to 7 (Croxall *et al*., 1952).

Sample collection and culturing of two isolates of Venturia inaequalis

We collected two samples of apple leaves with symptoms of *V. inaequalis*, one from a commercial orchard and the other from untreated apple trees. The commercial orchard sampled was located in Ifrane province (33° 24' 52.35"N, 5° 17' 30.84"W) and non-commercial apple trees, in the National School of Agriculture of Meknes (33° 50' 34.70"N, 5°28' 35.22"W). Apple leaves (Fig. 1A) with visible symptoms of *V. inaequalis* were packed in bags and kept at 4 °C for 24 h in the laboratory before beginning isolation of the pathogen.

In order to obtain isolates, we prepared potato dextrose agar medium (PDA) by peeling and boiling 250 g of potato tubers. After boiling, 700 ml of the liquid was filtered and complemented with autoclaved distilled water to obtain one litre of solution. Thereafter, 15 g of anhydrous glucose and 20 g of agar was added to the solution. The solution underwent magnetic agitation on a hot plate and was autoclaved at 121 °C for 20 min. At room temperature, the antibiotics chloramphenicol (50 µg/ml) and acetic acid (2 ml/l) were added to the PDA medium so that the two isolates would not be contaminated.

Isolation of *V. inaequalis* was performed in aseptic conditions under the laminar flow cabinet, which had been previously disinfected. To do this, we cut the leaves showing visible symptoms of *V. inaequalis* into pieces with sterilized scissors. Two leaf pieces were then dipped in 70% ethanol for 30 sec and then rinsed three times with autoclaved distilled water. Two leaf pieces were deposited at the opposite ends of a Petri dish containing PDA and kept at 23-25 °C. The growth of *V. inaequalis* obtained after 7 days of inoculation was then sub-cultured to get pure ones.

Choice of fungicides

In addition to protective fungicides, apple farmers in Morocco rely on three classes of systemic fungicides, namely, methyl benzimidazole carbamate (MBC) fungicides, demethylation inhibitors (DMIs) and the quinone-outside inhibitors (QoI). The three most widely used systemic fungicides that belong to these classes are thiophanate-methyl, difenoconazole and trifloxystrobin, respectively (Moinina *et al*., 2018). Precisely, we used the three aforementioned active ingredients. The tested concentrations for each of the active ingredients were 0.05, 0.5, 1.0, 5.0 and 10.0 µg/ml for both *in vitro* and *in vivo* tests. The sensitivity of *V. inaequalis* mycelial growth to these fungicides at recommended rates were evaluated on two isolates, one collected from a commercial apple orchard (ViIF) and the other from untreated apple trees (ViEN).

Determination of half-maximal effective concentration (EC_{50})

The dishes were incubated at 24-25 °C and the diameter of the resulting mycelial cultures was measured after 12 days. We repeated the experiment twice over in four replicates for each combination fungicide-concentration. In order to assess the sensitivity level of the tested isolates of *V. inaequalis*, radial mycelial growth (mm) was recorded with the aid of a digital Vernier Caliper for each dish when the uncontaminated controls fully covered the Petri dish. The percent fungal growth inhibition was calculated according to Pandey *et al.* (1982):

Inhibition rate (%) = [(a-b)/a] × 100 (2)

where: a-growth in the control; b-growth in the sample.

For each isolate, linear regression analysis was conducted using percent inhibition of colony growth and concentration for the respective fungicides to determine the value of the effective concentration that inhibited isolate growth by 50% (EC_{50}).

In vivo test of fungicide efficacy against V. inaequalis on detached leaves

We collected young apple leaves of 'Golden Delicious' cultivar with no visible symptoms of disease at the experimental site of the National School of Agriculture of Meknes. The protocol of pathogenicity by Nicholson *et al.*(1973) for testing of *V. inaequalis* by detached leaf assay was carefully implemented.

The experiment contained two treatments, looking at both the preventive and curative properties of the fungicides examined. For both approaches, detached leaves were submerged immediately in water. Thereafter, the leaves were gently rubbed to remove leaf hairs and debris and rinsed under running tap water for 15 min. The petiole closest to the leaf base was cut at an angle to allow maximum vascular contact with water in the Petri dish. The leaves were then rinsed three times with sterile water. The leaf was then placed adaxial side up in the Petri dish to ensure contact of the cut petiole with the water agar (1.2%).

For the preventive treatment, we first dipped the leaves in autoclaved distilled water amended with fungicide at a tested concentration for about two minutes. The treated leaves were then placed in Petri dishes with water agar medium for roughly two hours and allowed to dry before being inoculated with a conidial suspension of *V. inaequalis*.

For the curative treatment, the leaf was placed adaxial side up in the Petri dish to ensure contact between the cut petiole and the water agar. The upper leaf surface was kept dry in Petri dishes and placed in a laminar flow hood before dropping conidial suspension. Two hours later, a specific concentration of each fungicide was sprayed on each leaf in a Petri dish. 50 µL of conidial suspension (1×10^5 conidia/ml) was dropped on the adaxial surface of each leaf for both treatments. There was no fungicide application applied to the leaf surface of the control. Two replicates each containing two apple leaves per Petri dish was made for each concentration tested. Petri dishes were then sealed and stored under bright fluorescent light with 16 h light at 20 °C in a growth chamber. For both treatments, there were two experiments over time in four replicates (four leaves for each fungicide concentration).

Disease severity was determined 28 days after inoculation when the control was almost completely infected with *V. inaequalis*, by visually assessing the percentages of leaf area infected. The percentage of disease development of the leaves was rated on a 1-7 scale developed by Croxall *et al.*(1952) where 1 = 0% < percentage of scabbed leaf surface (S) < 1%; 2 = 1% < S < 5 %; 3 = 5% < S < 10%; 4 = 10 < S < 25%; 5 = 25% < S <50%; 6 = 50% < S < 75%; 7 = 75% < S. The fungicide efficacy was calculated using Abbott's formula (Abbott, 1925),

Efficacy (%) = (X-Y)/X ×100 (3)

Where X is the disease severity of the control and Y is the disease severity of the treatment.

Statistical analysis

The linear regression equation (Y= % inhibition; X = concentrations) and the EC_{50} value was derived from the line of best-fit. The resistance factor (RF) for each isolate was calculated as the ratio between the EC_{50} value of the fungicide treated commercial orchard sample and the EC_{50} value of the untreated apple tree sample (Kunz *et al.*, 1997). Analysis of variance (ANOVA) was used to determine the effects of fungicides and concentrations on the isolate and was carried out for both the *in vitro* and *in vivo* methods, and specific differences of disease severity were identified with Duncan's multiple range test using SPSS statistical analysis software (Version 20.0). Student test with two-sample assuming unequal variances with alpha value at 0.05 were performed for field survey.

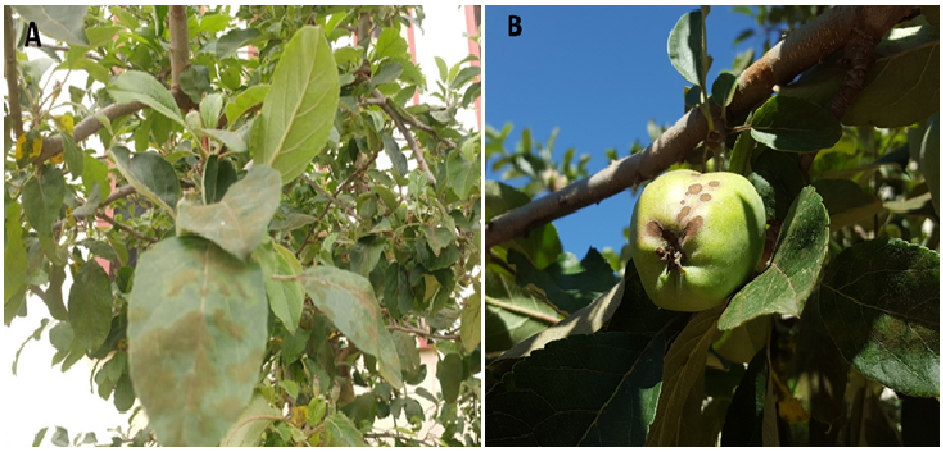

Fig. 1. Symptoms of *V. inaequalis*, causal agent of apple scab, on leaves (A) and fruit (B) of apple tree

Results

Incidence and severity of apple scab in seven districts of the Sais region

Apple scab incidence and severity in the region was recorded in all the districts. The results from the survey showed that among the three cultivars, 'Golden Delicious' cultivar was relatively the most susceptible to apple scab with the mean incidence of 40.31%, followed by 'Starking Delicious' (20.45%) and 'Gala' (18.92%) (Table 1). The highest mean of the incidence of apple scab appeared in Sidi El Makhfi community (40.91%) of Ifrane province. In the two districts of Sefrou province, Ait Sbaa and Laanoucer, the incidence was 30.70% and 15.97% respectively. The least incidence was observed in Ait Naamane (14.10%) of El Hajeb province (Fig. 2). There was a strong correlation between apple scab incidence and its severity in the surveyed region (r = 0.83). The grand mean of the disease incidence showed statistically a significant difference between the cultivars. Furthermore, there was a difference in the severity of the disease among the three cultivars. The lowest severity was observed in the orchards with 'Gala' cultivar (8.75%). The severity of apple scab was highest in Ain Leuh of Ifrane province reaching the rate of 20.71% of the surface leaf area affected. The least severity was in Dayet Aoua and Laanoucer with the rate of 5.71%. There were significant differences in the disease incidence and severity among the districts.

In vitro sensitivity testing

Sensitivity of Venturia inaequalis isolates to fungicides

After 12 days of incubation, percent inhibition of mycelial growth for each isolate was determined for each fungicide and concentration. A high variation in the sensitivity of each of the isolates of *V. inaequalis* was observed at all tested concentration levels. Difenoconazole had relatively different inhibitory effects on each of the two isolates. The inhibitory effect of this fungicide ranged from 9.43 to 70.94% for the isolate ViEN at the concentrations from 0.05 µg/ml to 10.00 µg/ml (Fig. 3A) and 13.42 to 85.00% for the isolate ViIF (Fig. 3B). According to the linear regression equations of the two isolates, the isolate ViEN from the untreated apple trees showed a lower sensitivity to difenoconazole and reflected a lower activity of this fungicide when compared to the isolate ViIF. There was a positive correlation between percent inhibition of the two isolates (r = 0.927, $P = 0.023$).

Trifloxystrobin had the greatest efficacy of the tested fungicides, inhibiting growth of the isolate ViEN from 13.57 to 91.44% (Fig. 3A) with concentrations from 0.05 µg/ml to 10.00 µg/ml respectively. It also had the greatest effect on isolate ViIF, inhibiting growth from 10.10 to 92.63% (Fig. 3B) with concentrations from 0.05 µg/ml to 10.00 µg/ml respectively.

Table 1. Prevalence of apple scab in the seven districts of the Sais region

Community	Golden Delicious		Gala		Starking Delicious	
	Incidence (%)	S (%)	Incidence (%)	S (%)	Incidence (%)	S (%)
Ain Leuh	40.00	20.00	15.00	5.71	55.00	37.14
Ait Naamane	35.33	24.00	1.11	1.90	0.00	0.00
Ait Sbaa	42.00	20.86	31.90	18.37	26.67	14.86
Dayet Aoua	35.83	8.57	3.33	3.81	0.00	0.00
Laanoucer	19.74	6.59	14.44	4.76	8.00	4.57
Sidi El Makhfi	55.00	32.50	33.33	12.38	32.50	22.86
Tigrigra	54.29	25.71	33.33	14.29	20.95	17.14
Grand mean	40.31[a]	19.75[b]	18.92[b]	8.75[c]	20.45[b]	13.80[b]

Percentages followed by the same letter do not differ significantly by Student t test at P=0.05
S: Percentage of affected leaf area

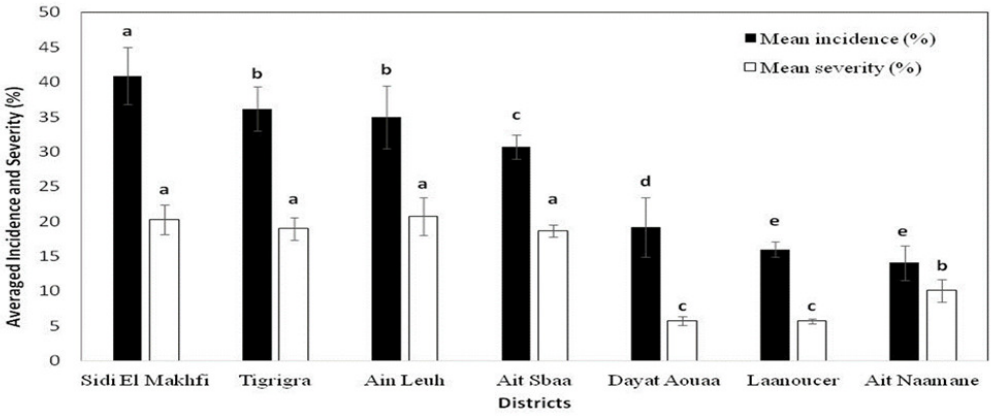

Fig. 2. Mean incidence and severity of apple scab in the different districts of the Sais region. Values marked with the same letter(s) are not statistically different (P ≤ 0.05)

The isolate ViIF was slightly less sensitive to trifloxystrobin than isolate ViEN, however there was a positive correlation between the two isolates (r = 0.985, P = 0.002).

Thiophanate-methyl was the least effective at inhibiting mycelial growth of both isolates. At the same concentrations as the previously tested fungicides, growth of the isolate ViEN was inhibited between 9.43 and 70.61% (Fig. 3A). The growth of the isolate ViIF was inhibited between 11.75 and 59.05% (Fig. 3B). The lack of inhibition at the highest concentration of thiophanate-methyl reflected a loss of sensitivity of the isolate ViIF compared to ViEN. However, there was a positive correlation between the inhibitory effects of the two isolates (r = 0.957, P = 0.011).

Determination of EC_{50} and resistance factor for the tested fungicides

After evaluating the mycelial growth of the pathogen, *V. inaequalis,* in each Petri dish, linear regression for each fungicide was established using fungicide concentrations and mycelial growth inhibition values (Fig. 3). For thiophanate-methyl, the regression equation was determined (y = 4.4045x + 17.593, R^2 = 0.869) and the fungicide concentration corresponding to 50% inhibition was calculated as 7.40 µg/ml for the isolate from the commercial apple orchard, ViIF. The same procedure was established for the other fungicides as well. EC_{50} values were recorded for the isolates ViEN and ViIF were 5.84 µg/ml and 3.24 µg/ml, and 2.33 µg/ml and 2.37 µg/ml for difenoconazole and trifloxystrobin, respectively (Table 2). The RF was calculated for the two isolates and the sensitivity of *V. inaequalis* isolate ViIF to difenoconazole was (RF = 0.55), which was lower than the RF values found for thiophanate-methyl (RF= 1.79) and trifloxystrobin (RF = 1.02). Although trifloxystrobin had a lower RF value than thiophanate-methyl, a concentration of 10µg/ml of trifloxystrobin was able to inhibit completely the mycelial growth of the isolates.

A three-way ANOVA showed that there was no significant difference in the inhibitory effects between the two *Venturia* isolates (P > 0.05). However, there were very high significant differences among treatments (P = 0.0001) and concentrations (P = 0.0000) on percent inhibition of *V. inaequalis* mycelial growth. There was no statistically significant interaction between treatments and concentrations in the inhibition of *V. inaequalis* mycelial growth (P = 0.0641) however, the interaction between Venturia isolates and treatments was highly significant (P = 0.0005) (Table 4).

In vivo evaluation of fungicide efficacy on detached apple leaves

Fungicide sensitivity was determined for the same concentration range on detached apple leaves after 28 days of incubation. Disease severity differed for each isolate based on the concentration and fungicide used. There were significant differences in disease severity on leaves between the active ingredients (treatments) in both the preventive

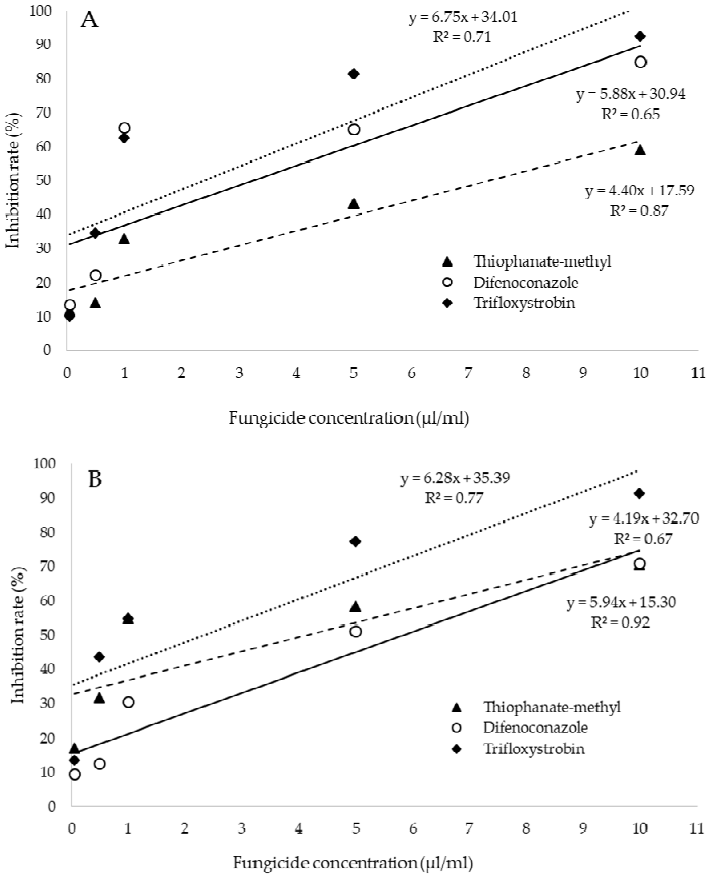

Fig. 3. Effect of three fungicides on the mycelial growth of the two isolates of *V. inaequalis* ViIF (A) and ViEN (B)

Table 2. Sensitivity of *V. inaequalis* isolates to thiophanate-methyl, difenoconazole andTrifloxystrobin

Active ingredient	Isolate ViEN	Isolate ViIF	RF
	EC_{50} (µg/ml)	EC_{50} (µg/ml)	
Thiophanate-methyl	4.13	7.40	1.79
Difenoconazole	5.84	3.24	0.55
Trifloxystrobin	2.33	2.37	1.02

Table 3. Averaged disease severity of the two isolates of *V. inaequalis* on detached apple leaves (n = 4)

	Difenoconazole		Thiophanate-methyl		Trifloxystrobin	
	ViEN	ViIF	ViEN	ViIF	ViEN	ViIF
0.00	99.25±0.48^{a}	99.50±0.50^{a}	99.25±0.48^{a}	99.50±0.50^{a}	99.25±0.48^{a}	99.50±0.50^{a}
0.05	78.57±9.22^{ab}	85.72±14.26^{a}	99.38±0.63^{a}	71.43±13.04^{ab}	64.29±9.05^{bc}	52.50±3.37^{b}
0.50	75.00±8.16^{b}	57.14±11.39^{b}	50.00±2.33^{b}	40.71±19.11^{b}	50.00±4.98^{c}	56.79±5.60^{b}
1.00	85.72±8.25^{ab}	45.36±11.98^{b}	55.54±6.94^{b}	64.00±13.25^{ab}	64.29±5.08^{bc}	46.43±14.50^{b}
5.00	68.75±4.75^{b}	50.00±2.91^{b}	40.71±7.77^{bc}	65.72±3.50^{ab}	67.86±4.86^{b}	42.86±16.45^{b}
10.00	48.36±4.78^{c}	39.20±6.19^{b}	28.60±8.23^{c}	92.86±7.14^{a}	18.75±2.98^{d}	21.43±13.68^{b}
LSD $_{0.05}{}^{x}$	19.76	27.78	16.38	33.75	15.60	32.35
0.00	99.25±0.48^{a}	99.50±0.50^{a}	99.25±0.48^{a}	99.50±0.50^{a}	99.25±0.48^{a}	99.50±0.50^{a}
0.05	51.43±18.03^{b}	92.86±4.13^{a}	68.50±8.21^{ab}	68.22±8.66^{a}	96.43±3.57^{a}	71.43±11.66^{ab}
0.50	73.71±5.98^{ab}	70.53±15.18^{ab}	57.14±17.50^{b}	82.14±13.52^{a}	92.86±7.14^{a}	50.32±17.17^{b}
1.00	78.57±4.12^{ab}	50.00±17.97^{b}	30.86±14.04^{b}	75.00±14.73^{a}	72.50±15.11^{ab}	10.71±10.72^{c}
5.00	80.00±5.00^{ab}	75.00±8.99^{ab}	53.93±18.90^{b}	96.43±3.57^{a}	50.25±6.75^{bc}	3.57±3.57^{c}
10.00	75.00±16.88^{ab}	75.00±11.93^{ab}	62.25±6.25^{ab}	70.00±8.41^{a}	36.00±10.34^{c}	3.60±3.60^{c}
LSD $_{0.05}{}^{y}$	31.82	34.18	37.72	28.66	25.59	28.99

Means followed by the same letter in the same column are not significantly different according to Duncan's multiple range test (P = 0.05). Least significant difference (LSD) test at P = 0.05 of mean disease severity using ^{x} preventive method and ^{y} curative method

Table 4. Analysis of variance for the effects of *Venturia* isolates, fungicide treatments and concentrations on inhibition rate of mycelial growth and mean severity of detached leaves

Parameter and source of variation	df	Mean square	F *value*	*P*
1. In vitro sensitivity test (n = 4)				
Dependent variable: inhibition rate (%)				
Venturia isolate	1	1.233	0.033	0.8583
Treatment	2	802.042	21.657	0.0001
Concentration	4	4264.008	115.138	0.0000
Venturia isolate × Treatment	2	556.077	15.015	0.0005
Treatment × Concentration	8	97.252	2.626	0.0641
Error	119	37.034		
2. In vivo sensitivity test				
Dependent variable: mean severity (%)				
x. Preventive method (n = 4)				
Venturia isolate	1	684.870	1.460	0.2293
Treatment	2	2692.867	5.742	0.0042
Concentration	4	3200.164	6.824	0.0001
Error	119	468.984		
y. Curative method (n = 4)				
Venturia isolate	1	889.523	1.073	0.3024
Treatment	2	5968.732	7.199	0.0011
Concentration	4	2415.540	2.913	0.0243
Error	119	829.130		

and curative treatments (Table 4). With the curative treatment, specifically, disease severity on leaves treated with trifloxystrobin was much lower than that of the other active ingredients at a concentration of 10 µg/ml (Table 3). Mean disease severity of the control (0.00 µg/ml) of isolate ViEN from untreated apple trees (99.25%) was slightly greater than that of isolate ViIF from the commercial apple orchard (99.50%).

With the preventive treatment, the lowest disease severity was observed on leaves treated with trifloxystrobin. Disease severity ranged from 18.75 to 64.29% and 21.43 to 52.50%, for the leaves inoculated with isolates ViEN and ViIF, respectively. The fungicide resulted in a greater reduction of disease severity on isolate ViIF with the curative treatment, with a range from 3.57 to 71.43%. Moreover, disease severity was lower on leaves inoculated

with isolate ViEN collected from untreated apple trees with the curative treatment (Table 3). The isolate ViIF was more sensitive to trifloxystrobin compared to the other fungicides in both the preventative and curative treatments.

With thiophanate-methyl, the variation in disease severity in the two treatments was clearly observed. With the preventive treatments, disease severity ranged from 28.60 to 99.38% and from 40.71 to 92.86%, for the isolates ViEN and ViIF respectively. With the curative treatments, although disease severity was relatively higher than that of the preventive treatments, there was no significant difference between the mean of the two treatments. Leaves inoculated with the isolate ViIF had disease severity ranging from 36.00 to 96.43%.

In the case of detached leaves treated with difenoconazole, mean disease severity recorded by the preventive treatments ranged from 48.36 to 85.72%, and from 39.20 to 85.72%, for the isolates ViEN and ViIF, respectively. The lowest disease severity was recorded from leaves treated with the highest concentration of fungicide. With the curative treatments, disease severity was between 51.43 and 80.00%, and 50.00 and 92.86% for ViEN and ViIF, respectively.

In order to determine the efficacy of the fungicide treatment at a concentration of 10 µg/ml, we had to consider the severity of the control (0.00 µg/ml) and the corresponding severity at a concentration of 10 µg/ml for each fungicide and for each isolate from the commercial orchard. Trifloxystrobin had the highest efficacy, 78.46% and 96.38%, based on the preventive and curative treatments, respectively. Conversely, thiophanate-methyl was the least effective. Its efficacy was 6.67% for the preventive treatments and 29.65% for the curative treatments. In the case of difenoconazole, its efficacy was greater when used preventatively (60.60%) compared to when it was used as a curative treatment (24.62%).

According to the analysis of variance (ANOVA) of the sensitivity of *V. inaequalis* on detached leaves to fungicide application (Table 4), there were no significant differences between the two isolates in mean severity in either of the curative or preventive treatments. Fungicides applied as a curative treatment ($P = 0.0011$) showed higher significant effects than in preventive treatments ($P = 0.0042$) on mean disease severity. In other words, efficacy of fungicide treatments was significantly higher when used preventively compared to curatively. However, the effect of concentrations on mean disease severity on detached leaves was very highly significant in both the preventive ($P = 0.0001$) and curative treatments ($P = 0.0243$).

Discussion

The susceptibility of apple cultivars to apple scab and sensitivity of *V. inaequalis* isolates to fungicides were demonstrated for the first time in Morocco. In this study, the comparison of fungicide efficacy used an isolate (ViIF) collected from a commercial apple orchard located in Ifrane province and another isolate (ViEN) from untreated apple trees in the National School of Agriculture of Meknes. After several microscopic observations, the spores were bicellular and asymmetrical. Thus, average size of the ascospores was approximately 15 µm. According to Vaillancourt and Hartman (2000), the length of the ascospores varies between 12 and 15 µm, and the color is yellowish-green or brownish.

Disease survey. The visual method adopted in this study was carefully established in order to have a degree of accuracy as the more elaborate research method. Thus, Croxall *et al*. (1952) attest that the visual method of evaluation of apple scab infection gives a reliable result. In determining relative cultivar susceptibility, Biggs *et al*. (2010) fix a minimum of 10% scab leaf infection incidence on the cultivar. In this regard, all the three cultivars proved to be susceptible to apple scab. The susceptibility of the three cultivars to apple scab is known worldwide (Jha *et al*., 2010).

Knowledge on disease threshold, although not easily implemented, will help farmers confirm the efficacy of fungicide sprays. Visual monitoring of 20-50 trees per orchard of apple scab from green tip to fruit maturity stage will help reduce costs and fungicide inputs. The need for fungicide sprays during the summer is based on monitoring leaf scab incidence and using a threshold of 0.5 or 1.0% leaf scabbed (Carisse *et al*., 2009; Carisse and Jobin, 2012). A threshold of 7% scab incidence on developing fruit was proposed by Turechek and Wilcox (2005).

In vitro sensitivity testing. Efficacy of difenoconazole was lower on the isolate collected from untreated apple trees, ViEN, than the isolate collected from the commercial apple orchard, ViIF. The isolates insensitivity to the fungicides could be the result of several factors. For instance, EC_{50} values for difenoconazole were 5.84 µg/ml and 3.40 µg/ml for the isolates ViEN and ViIF, respectively (Table 2). At the same fungicide concentration range, Jobin and Carisse (2007) obtained a mean EC_{50} of 3.079 µg/ml for DMIs of the isolates obtained from commercial orchards. Similar results were recorded by Kunz *et al*. (1997) having obtained EC_{50} and RF values of 5.4 µg/ml and 59, respectively. Conversely, these values were greater than the ones reported by Villani *et al*. (2015) and Fiaccadori, (2017) who reported baseline EC_{50} values of 0.0075 and 0.002 µg/ml, respectively, and suggested that several isolates in the population were already insensitive to difenoconazole. The resistance factor obtained in our study was less than one. This showed that even though the EC_{50} values were highly influenced by the concentration range, the isolate from the commercial orchard was not very sensitive to the active ingredient. RF values documented by Mondino *et al*. (2015) were greater than five and with an EC_{50} value of 2.38 µg/ml under *in vitro* conditions.

The inhibitory effect of thiophanate-methyl was lowest compared to the other active ingredients. According to Quello *et al*. (2010), *V. inaequalis* isolates that can grow rapidly in 5 µg/ml of thiophanate methyl are classified as moderately resistant. Both of the tested isolates ViEN and ViIF, grew at this concentration. Even though the two isolates expressed sensitivity to trifloxystrobin, EC_{50} values were greater than 2 µg/ml Küng Färber *et al*. (2002) and Stević *et al*. (2015) obtained EC_{50} values less than 1µg/ml. According to Fiaccadori *et al*. (2011), isolates with EC_{50} values greater than 2 µg/ml and possessing the G143A substitution are considered resistant to trifloxystrobin.

Resistance or sensitivity of *V. inaequalis* to trifloxystrobin has yet to be confirmed in in Morocco.

In vivo sensitivity tests. Trifloxystrobin at a concentration of 10 µg/ml showed satisfactory efficacy in controlling disease severity for both preventive and curative treatments for both isolates of *V. inaequalis*. With a concentration of 10 µg/ml, the fungicide effectively reduced disease severity by 78.46 and 96.38% using the preventive and curative treatments, respectively. Comparatively, Stević *et al*. (2015) reported disease severity reduction of 31.0% when trifloxystrobin was applied at a concentration of 50 µg/ml.

At a concentration of 10 µg/ml, difenoconazole reduced disease severity of isolate ViIF by 60.60% with the preventive treatment and 29.65% with the curative treatment. The reduction in disease severity was greater than what was reported by Stević *et al*. (2010) who recorded a negligible a reduction in disease severity of 8.6% at a concentration of 30 µg/ml. The obtained results concur with the findings of (Köller *et al*., 1997; Kunz *et al*., 1997).

Inadequate disease reduction (6.67 and 29.65%, with preventive and curative treatments, respectively), was obtained when thiophanate methyl was used at a concentration of 10 µg/ml against ViIF (Table 3). This MBC fungicide was associated with high disease severity rates on both isolates at all concentrations tested.

The results presented above may not reflect the efficacy of fungicides under field conditions. This is supported by the fact that fungicide application during the in vivo tests was conducted 2 h before inoculation (preventive treatment) and after inoculation (curative treatment) with 1 x10^5 spores/ml applied to each of the detached leaves. The tested concentrations used in this study differ from recommended doses used in apple orchards. For instance, a dose of 15 ml/hl (150 ppm) of difenoconazole is recommended in field conditions (ONSSA, 2017). Optimal conditions for fungicide efficacy rarely occur under field conditions.

Conclusions

Disease survey showed the susceptibility of the three cultivars evaluated. In laboratory tests, there were no significant differences in fungicide sensitivity or mean disease severity between the two isolates of *V. inaequalis*. However, disease severity was significantly different among the fungicide treatments in both the preventive and curative treatments. The results presented are not quite representative of apple producing orchards as a limited number of isolates were used in this study. Furthermore, the results do not reflect the efficacy of fungicides under field conditions. For future research, we recommend using a larger number of isolates from various apple orchards. Contact fungicides should be tested in order to make comparisons with systemic fungicides on their efficacy against *V. inaequalis*. In addition, specific situations should be analyzed in detail to better understand the local response of *V. inaequalis* isolates to fungicides used to control the disease. Baseline sensitivity and molecular identification of races of *V. inaequalis* in Moroccan apple orchards will be of paramount importance.

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Conflicts of interest

The authors declare no conflict of interest and the funders have no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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